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Gopalan et al.

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(54) **TRANSGENIC LEGUMES**

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(51) **Int. Cl.**
C12N 15/82 (2006.01)

(52) **U.S. Cl.**
CPC *C12N 15/8271* (2013.01); *C12N 15/8241* (2013.01)

(58) **Field of Classification Search**
None
See application file for complete search history.

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(74) *Attorney, Agent, or Firm* — Isaac Estrada; Deborah Peacock; Peacock Myers, P.C.

(57) **ABSTRACT**

Embodiments of the present invention comprise altering the biosynthesis and accumulation of sucrose in legumes by transforming the plants with the sucrose phosphate synthase (SPS) gene of maize, and closely related regulatory genes. Embodiments of the present invention further comprise altering the assimilation of nitrogen in legumes by transforming the plants with the glutamine synthetase (GS) gene of soybean, and closely related regulatory genes. Embodiments of the present invention further comprise transforming legume plants with both SPS and GS genes. In addition, embodiments of the present invention relate to enhancing expression of transgenes through the 5' UTR of the *glycine max* (soybean) cytosolic glutamine synthetase (Gmglnβ₁) gene.
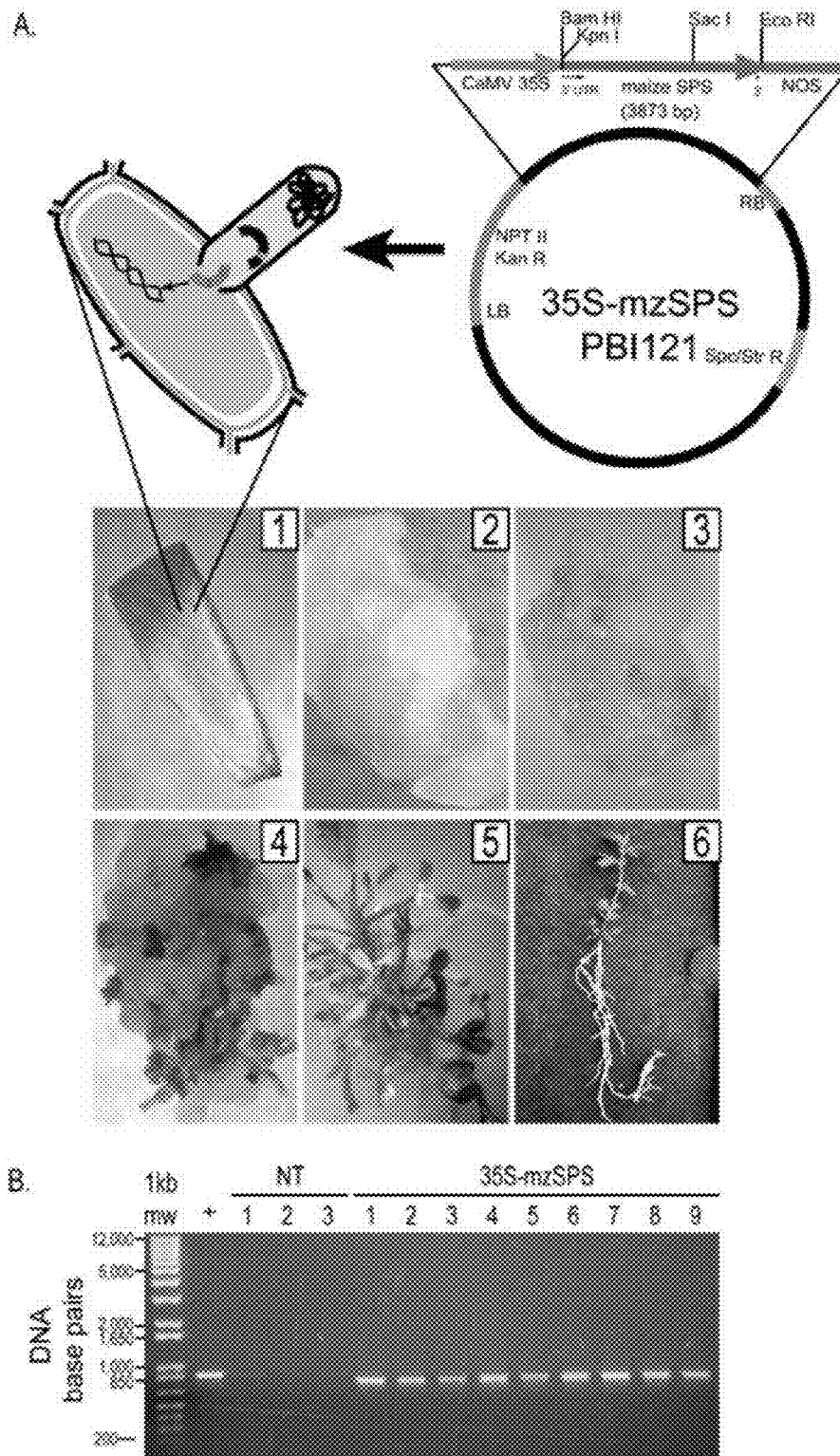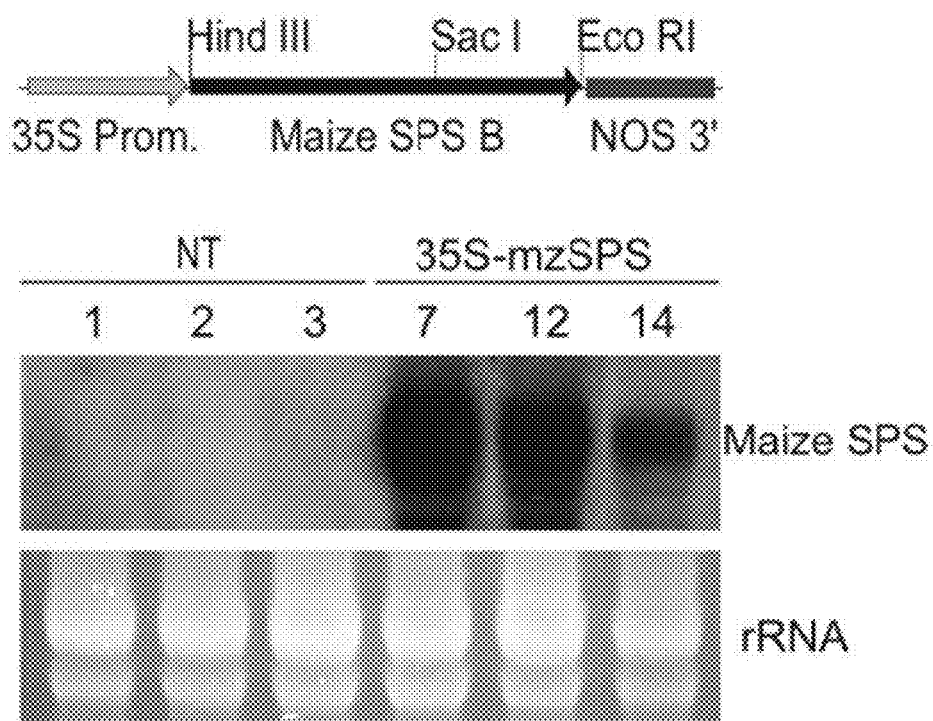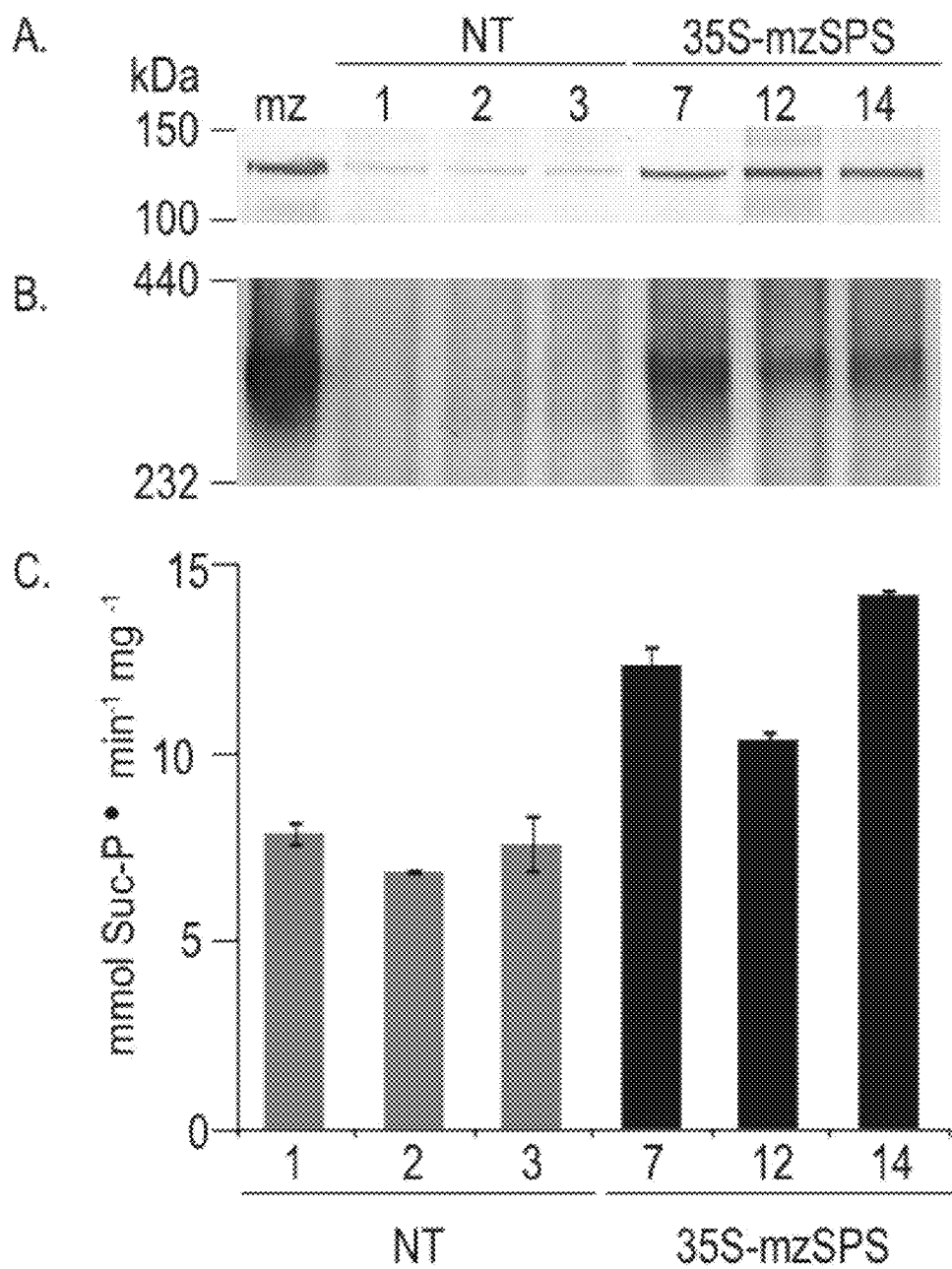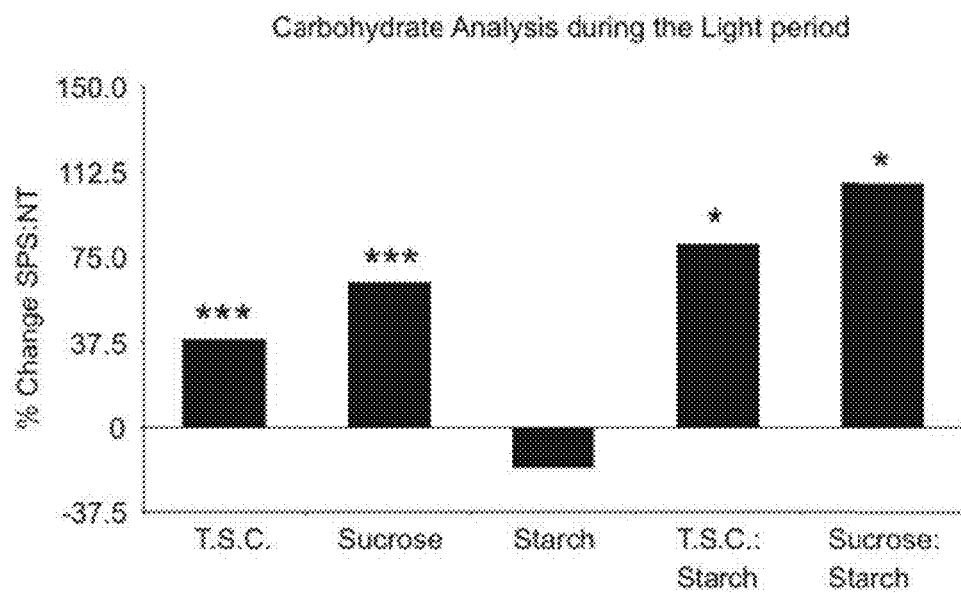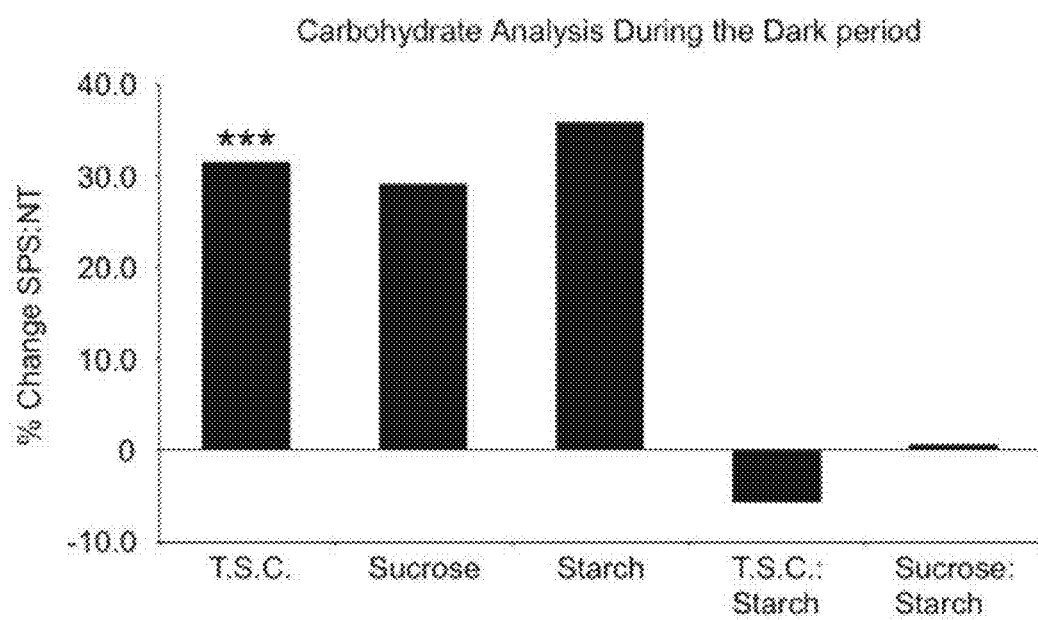
4 Claims, 38 Drawing Sheets

FIG. 1

FIG. 2

FIG. 3

FIG. 4

FIG. 5

FIG. 6

FIG. 7

FIG. 8

FIG. 9

Leaf Photosynthetic, Structural, and Chemical Characteristics.

FIG. 10

FIG. 11
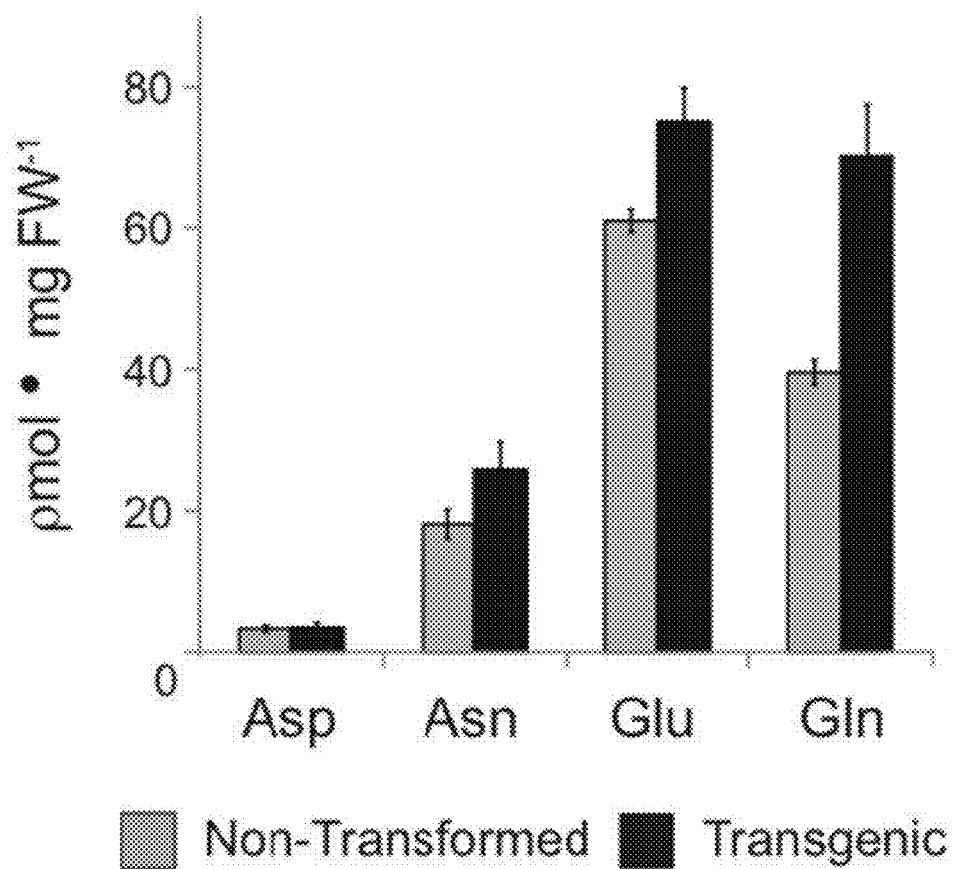
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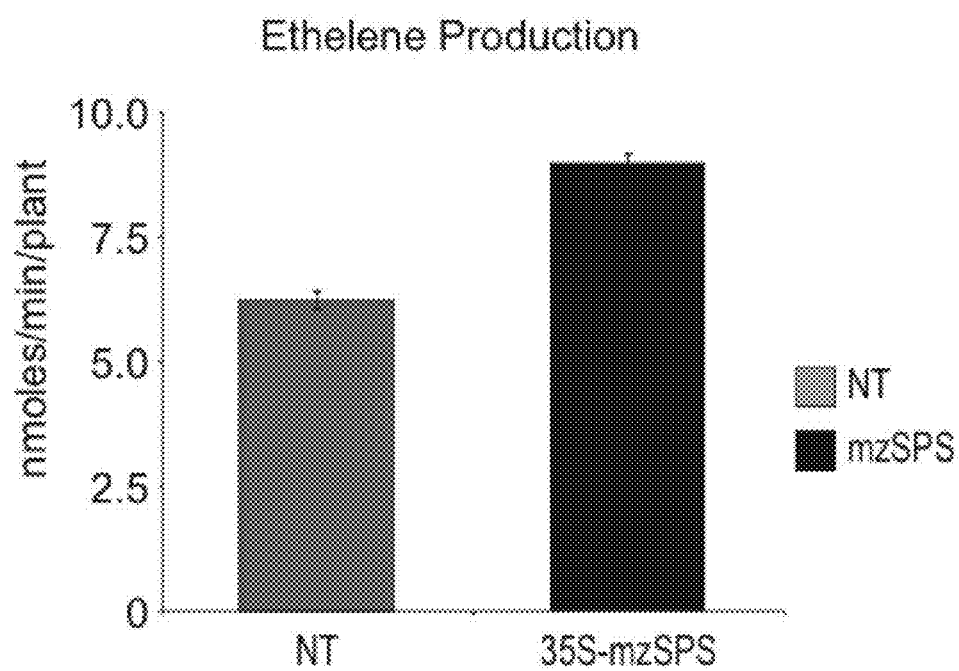
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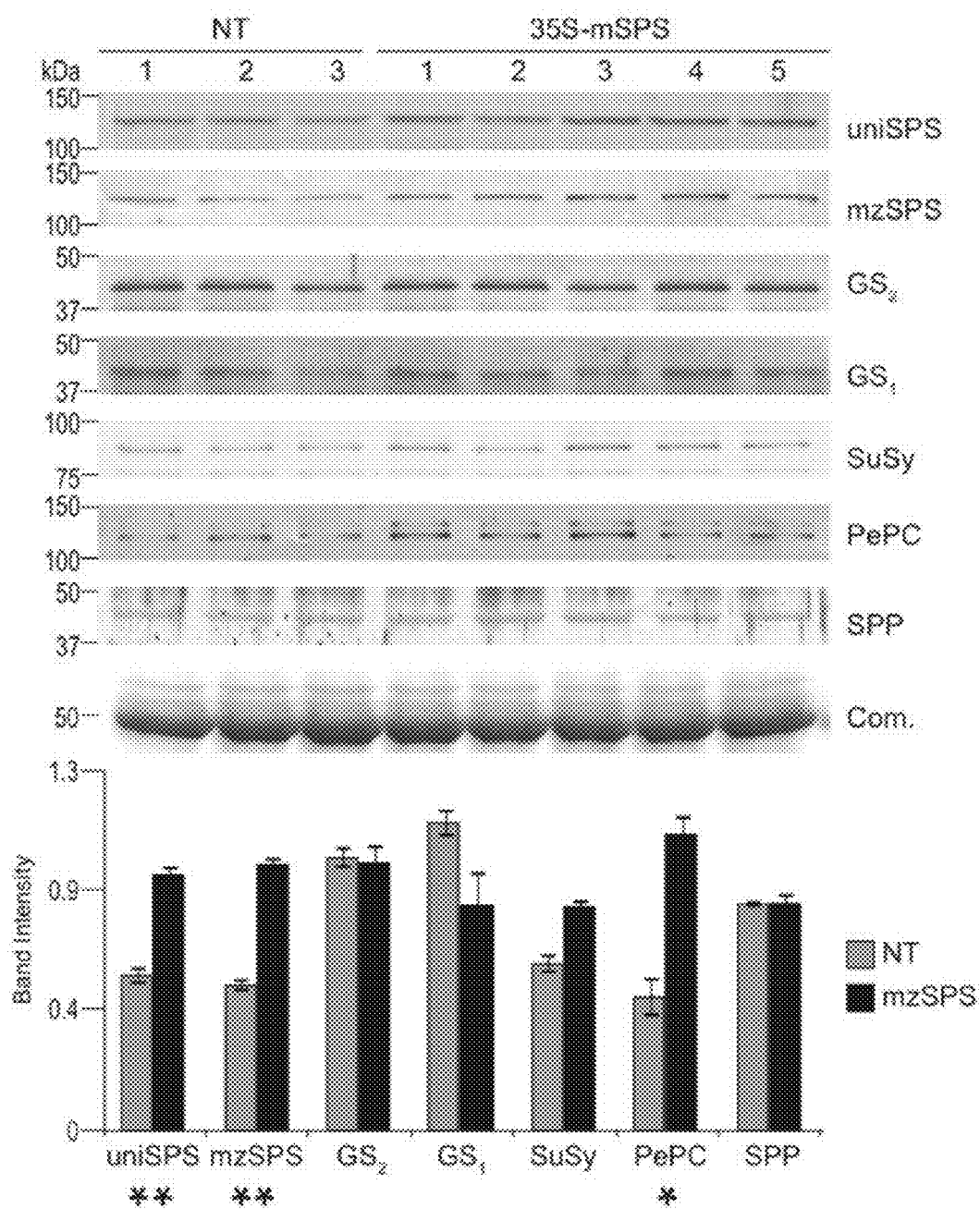
FIG. 14

FIG. 15

FIG. 16

FIG. 17

FIG. 18

A   Nitrogen and Protein Content Analysis

B   Fiber Content Analysis

FIG. 19

FIG. 20
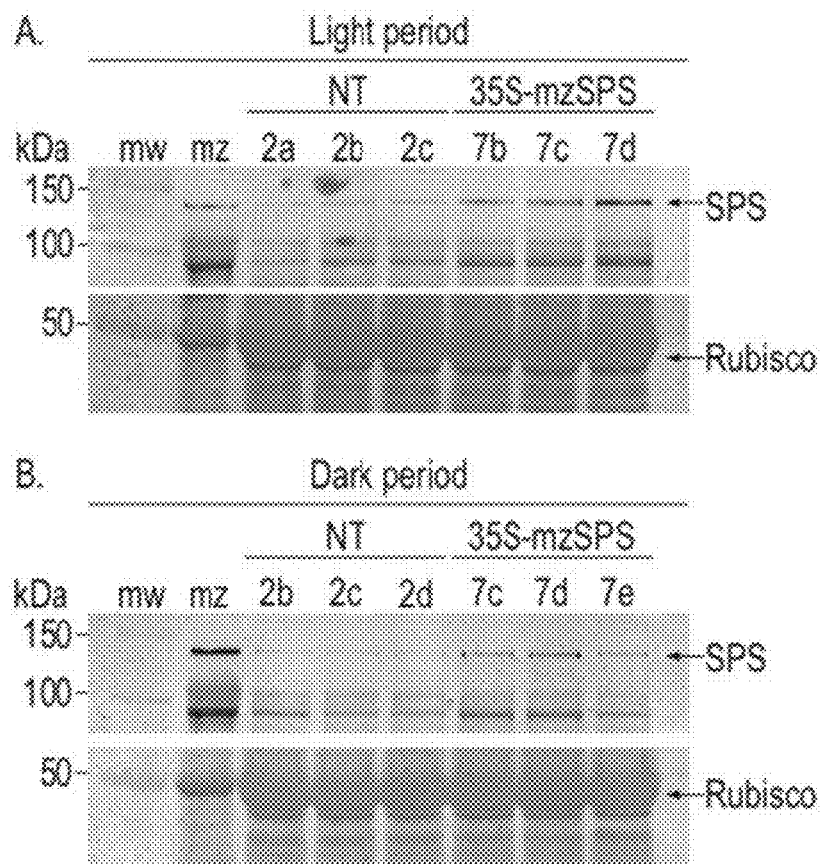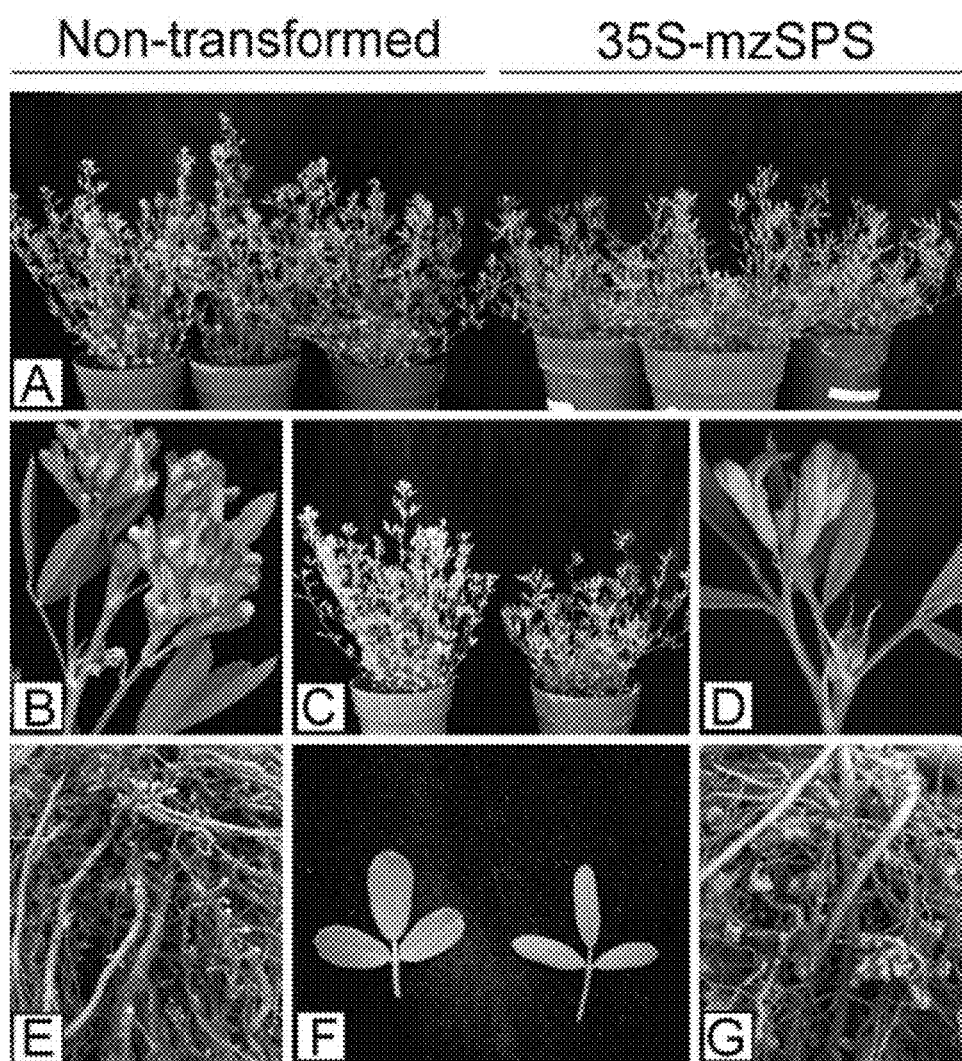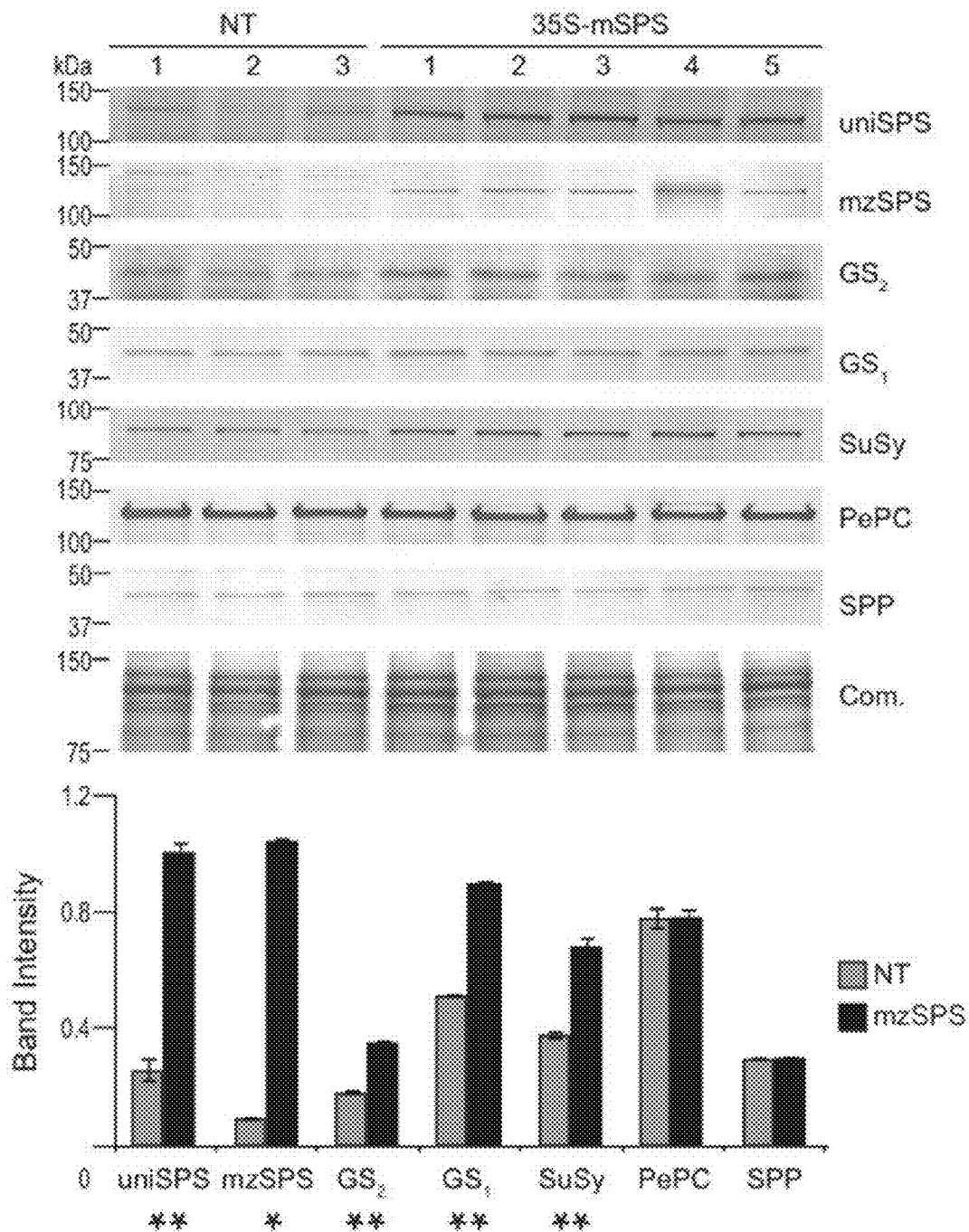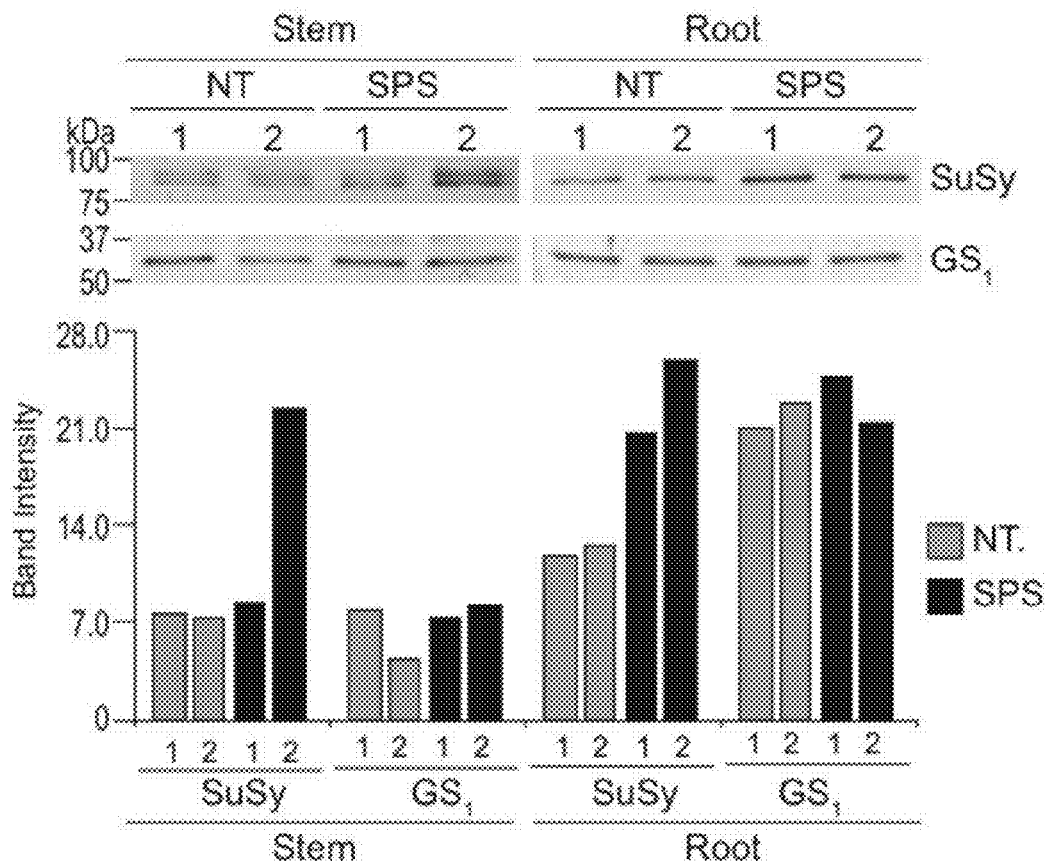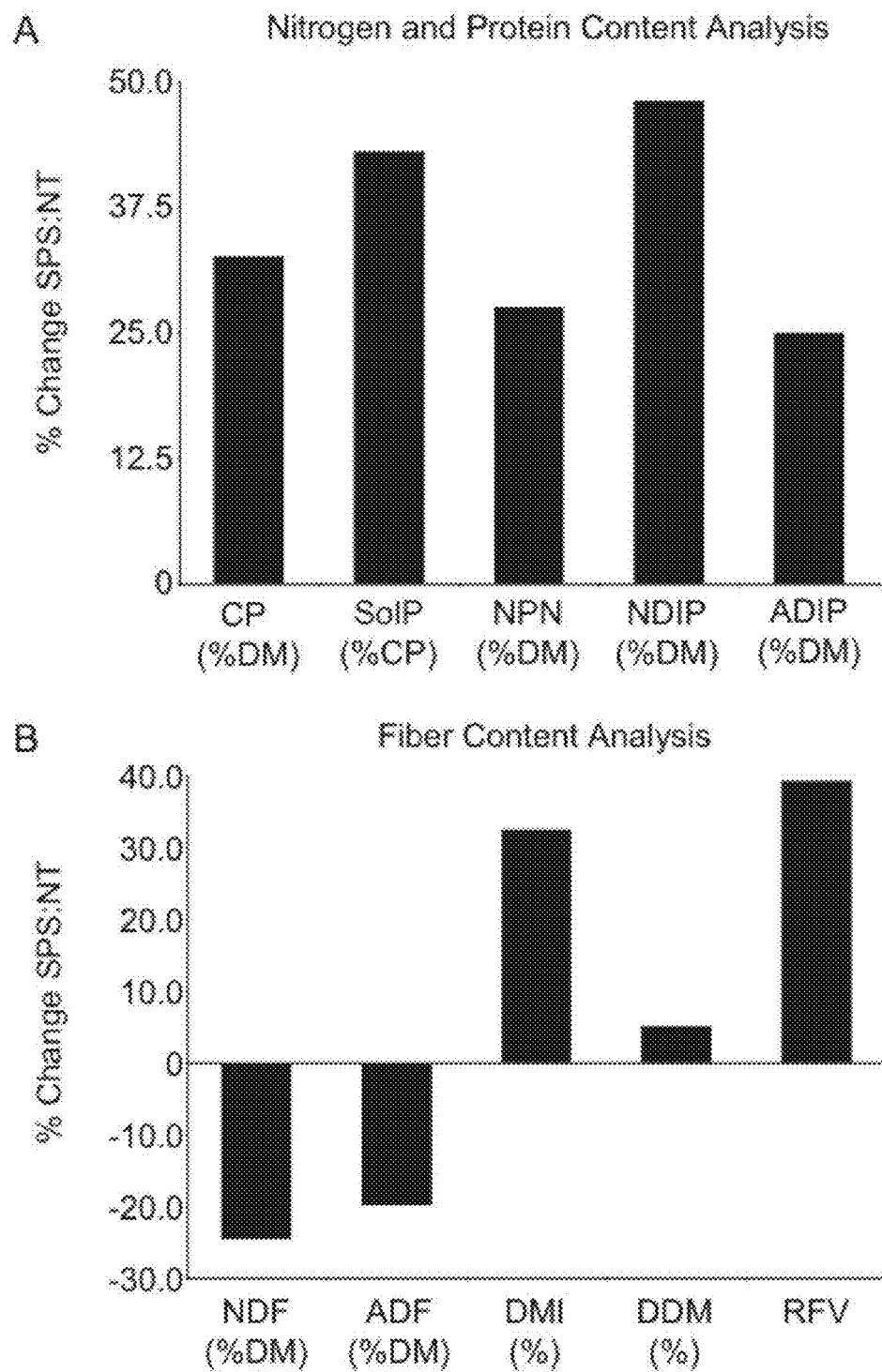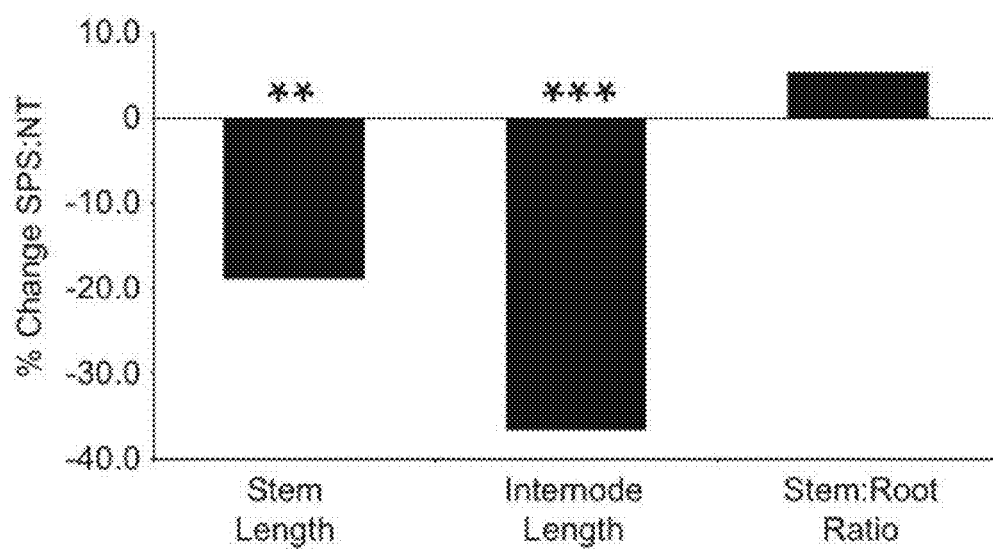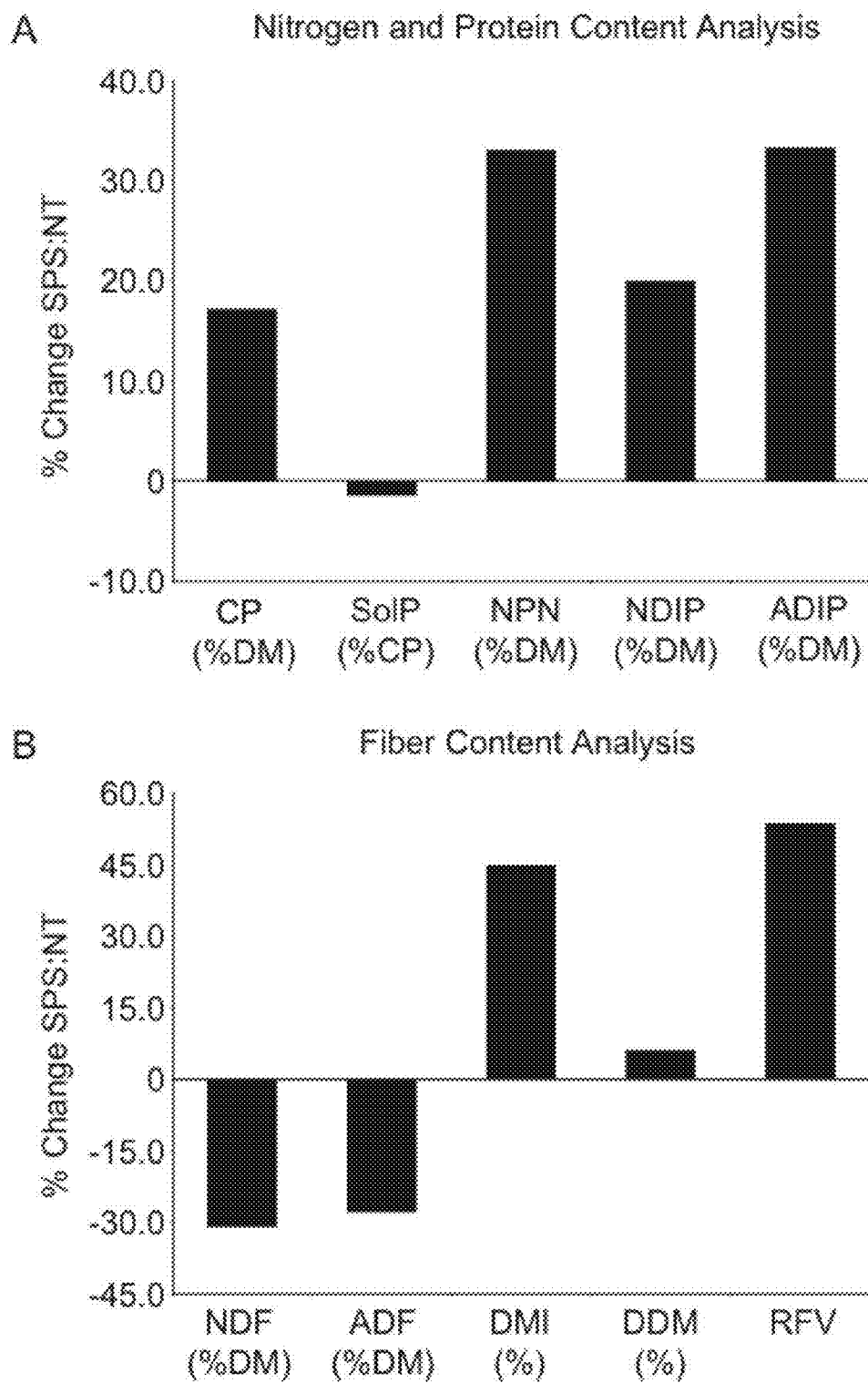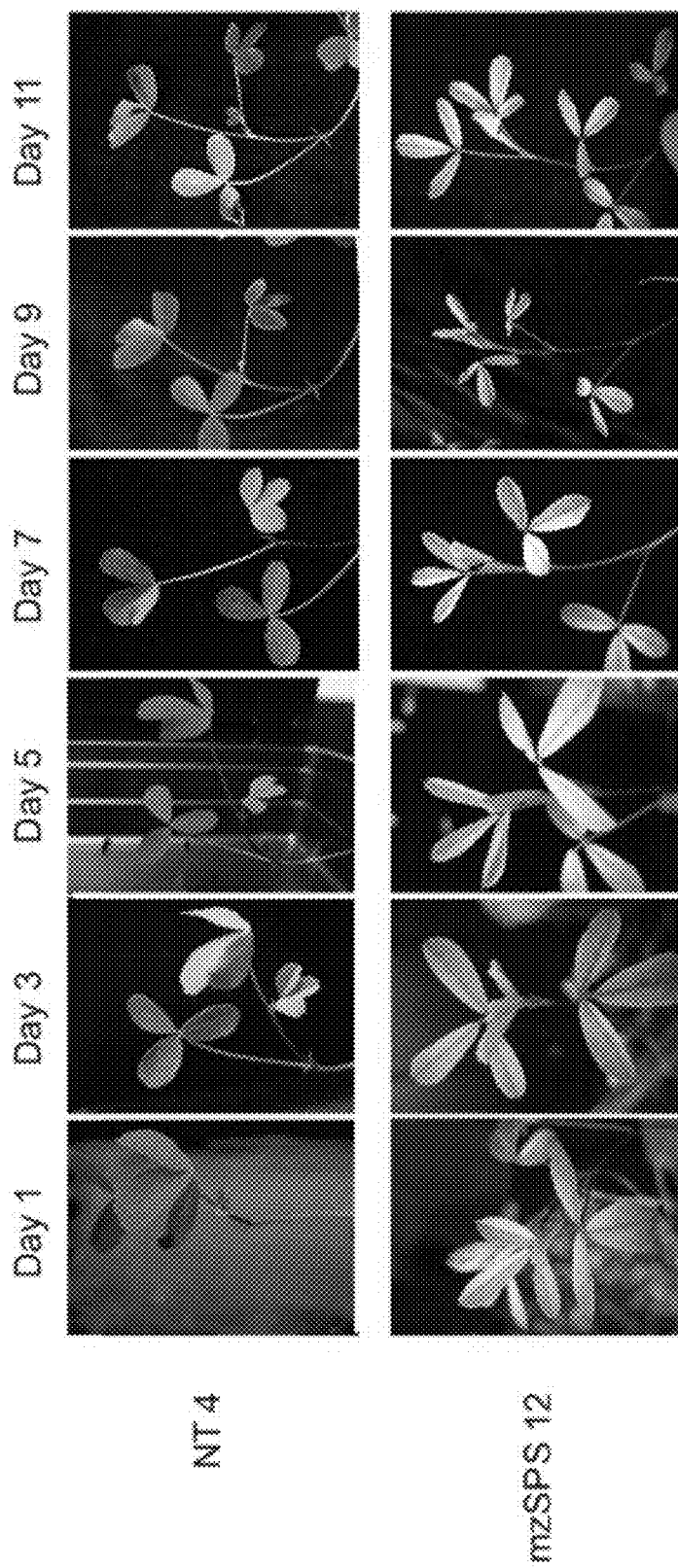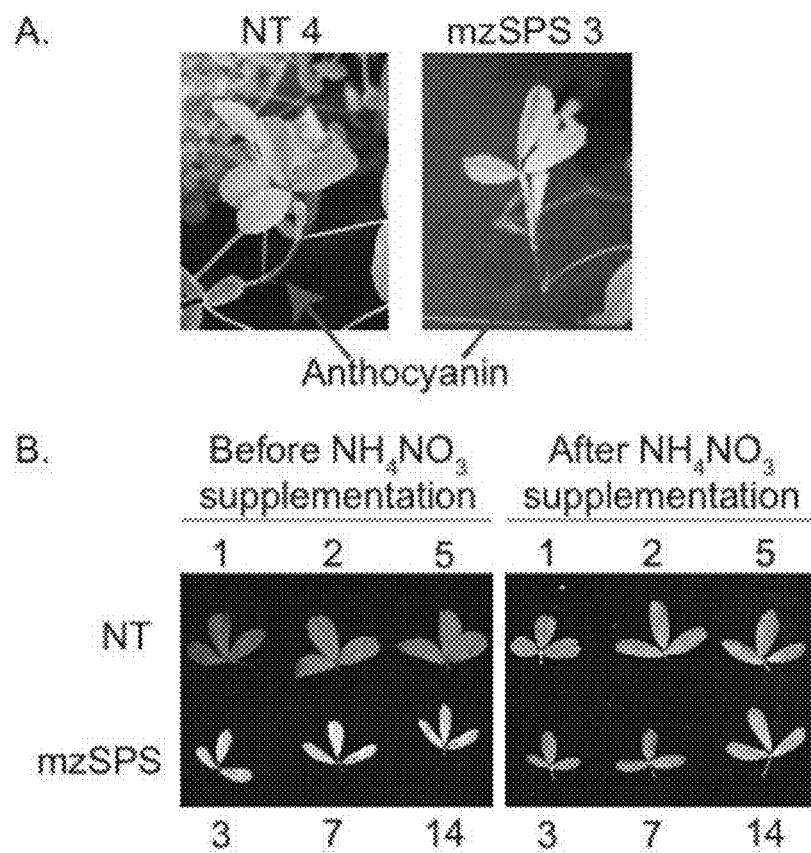
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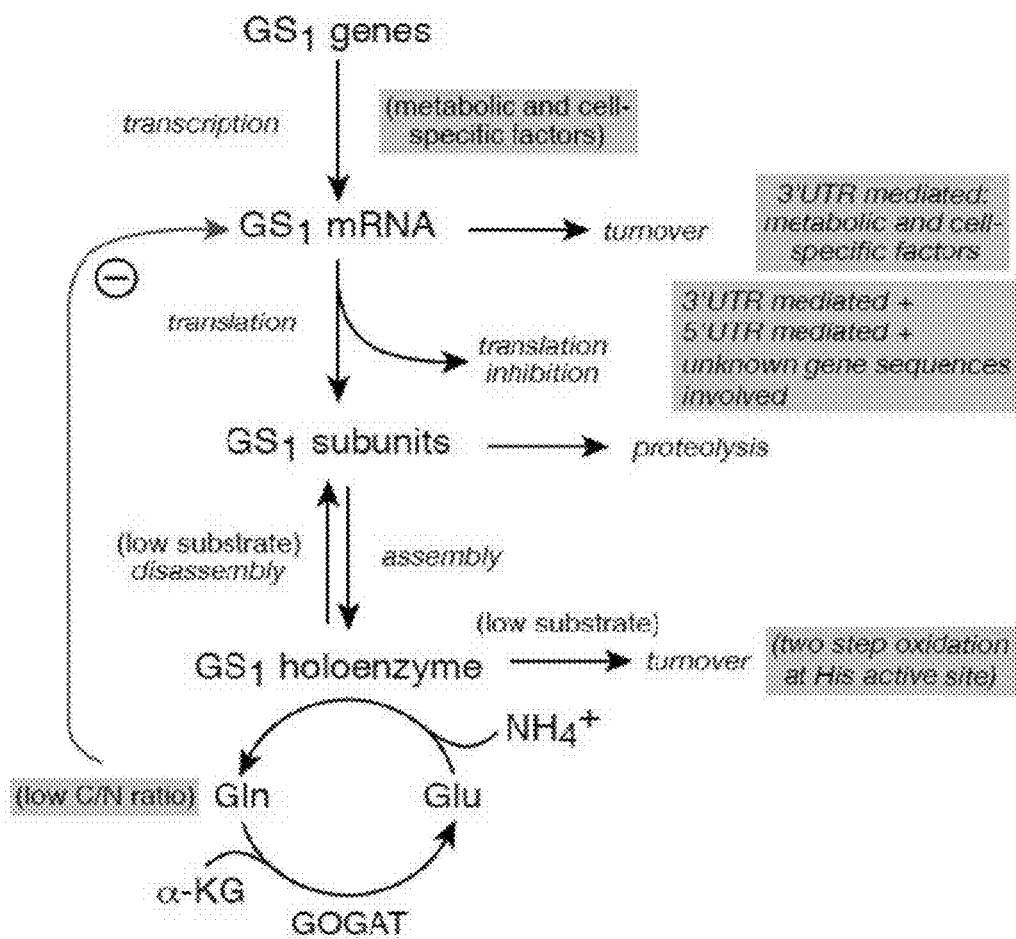
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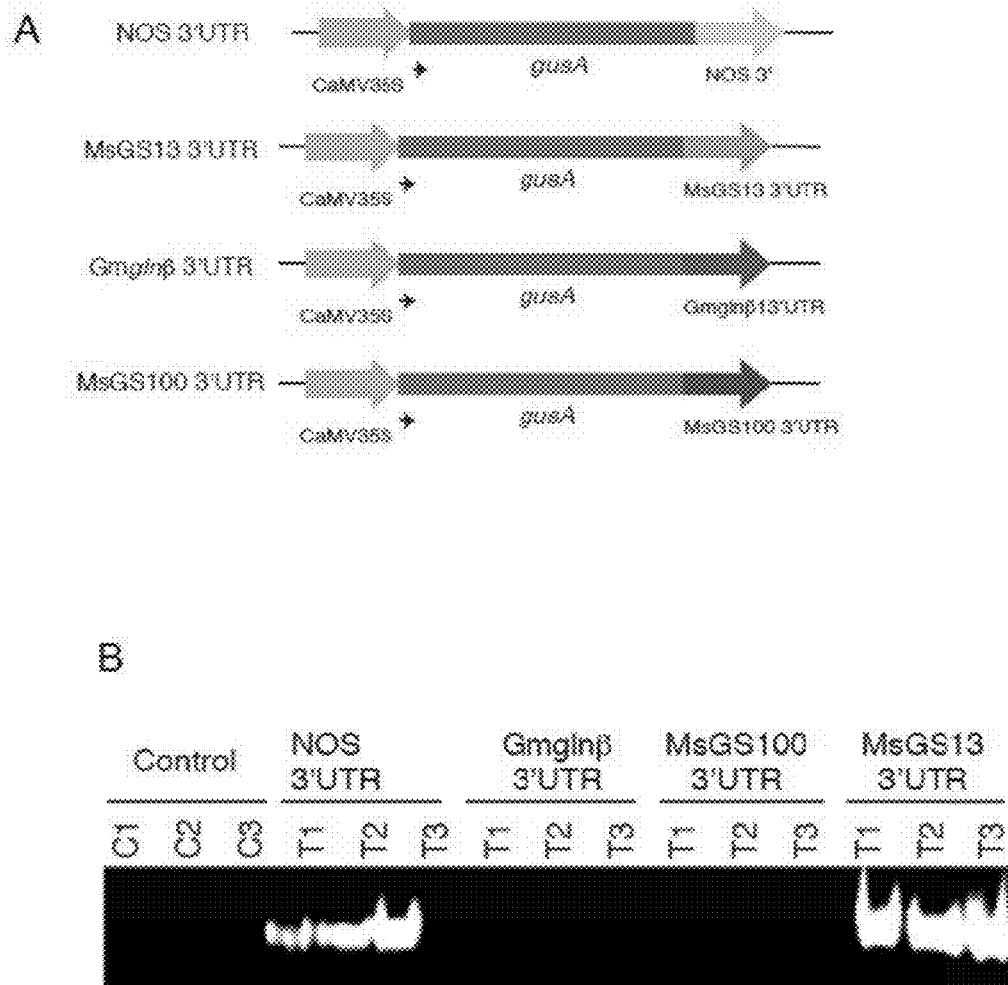
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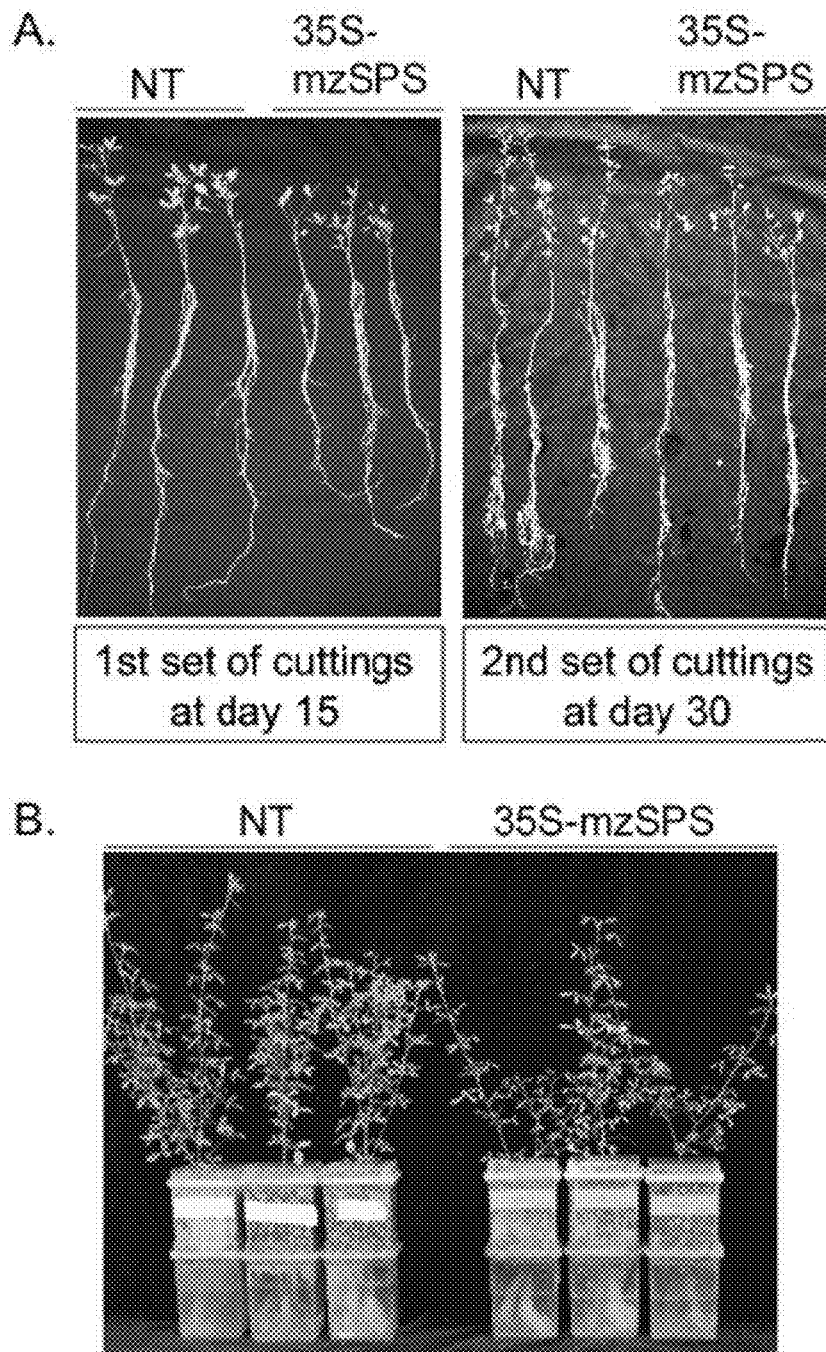
FIG. 24

A

B

FIG. 25

A

SEQ ID. NO. 16

Gmgfd81 3UTR

B
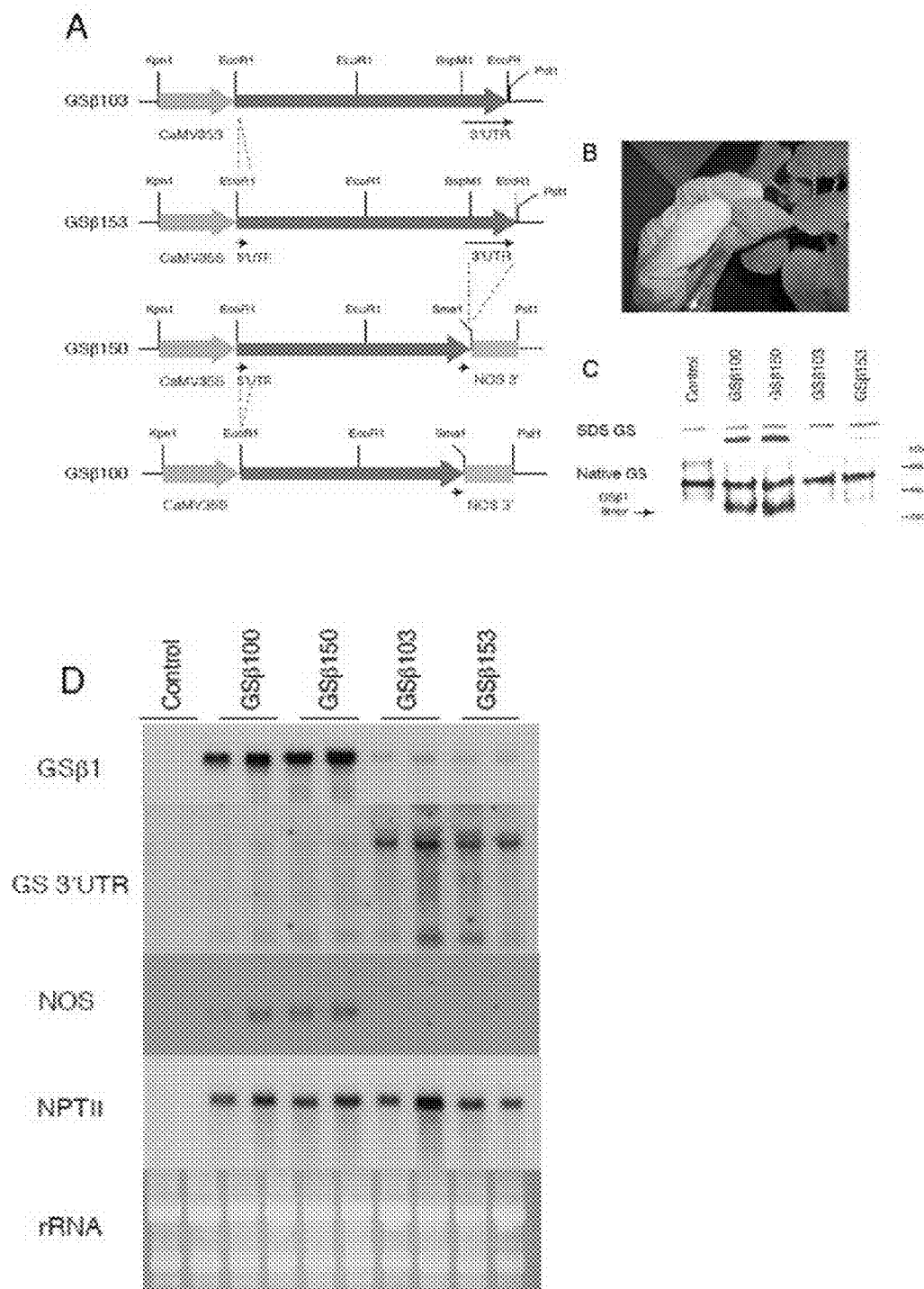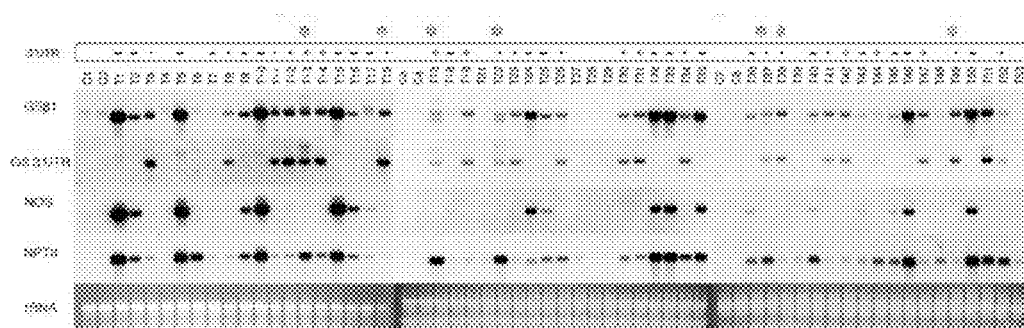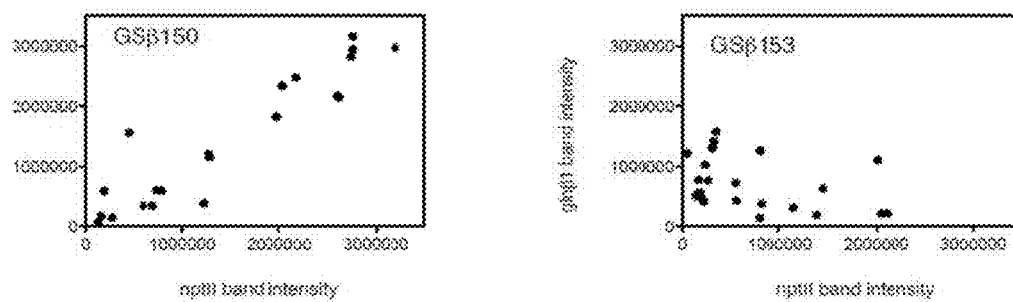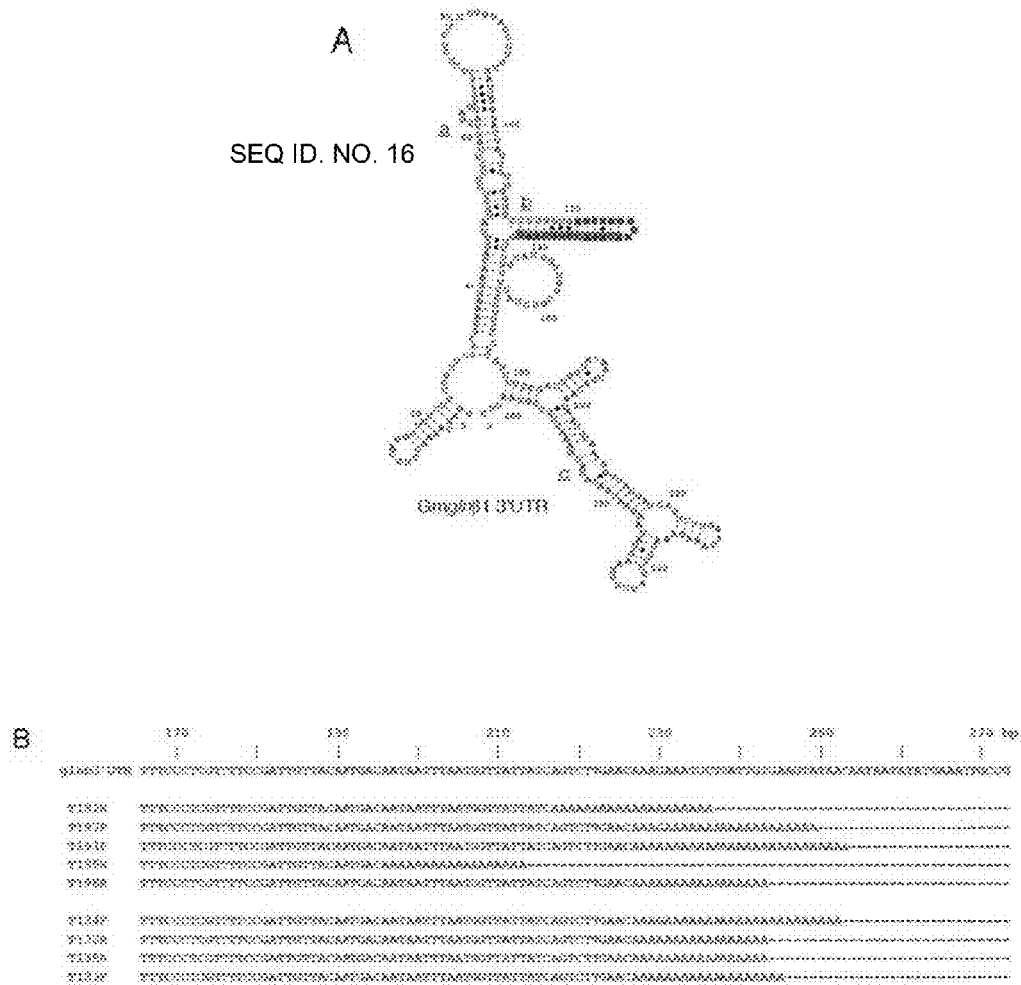
FIG. 26
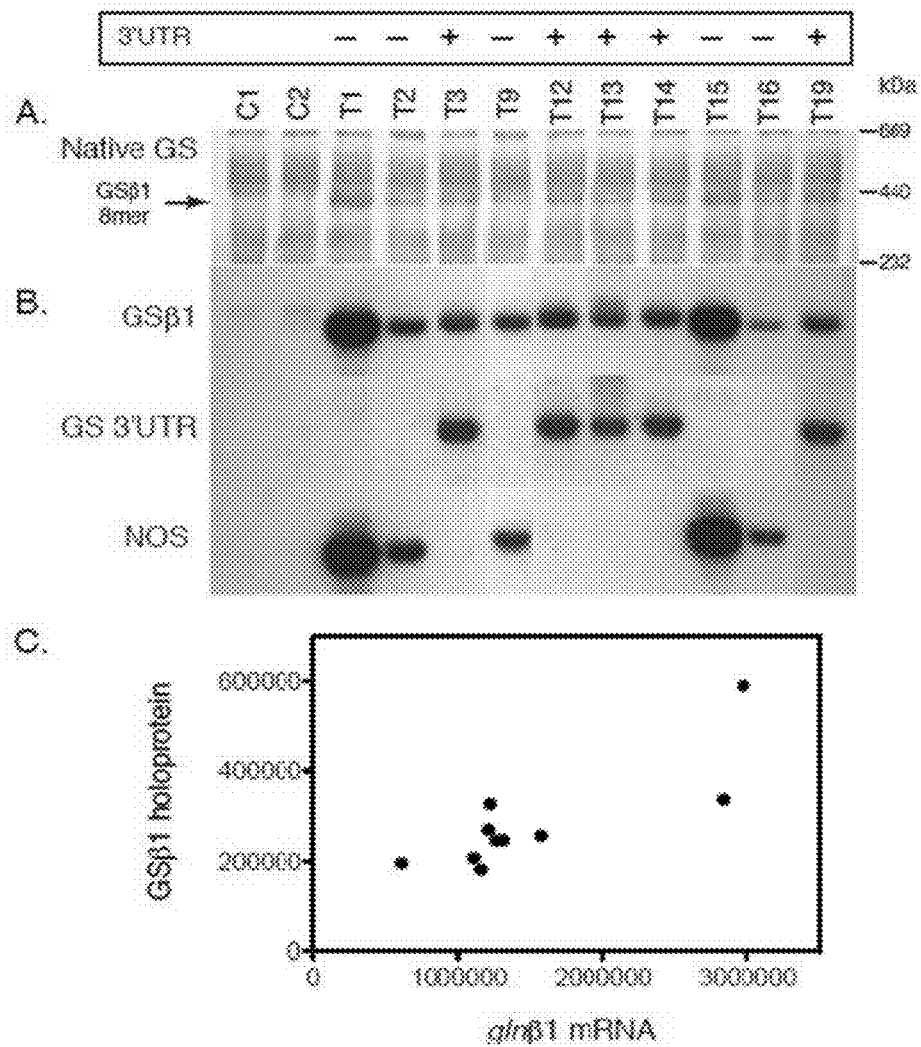
FIG. 27
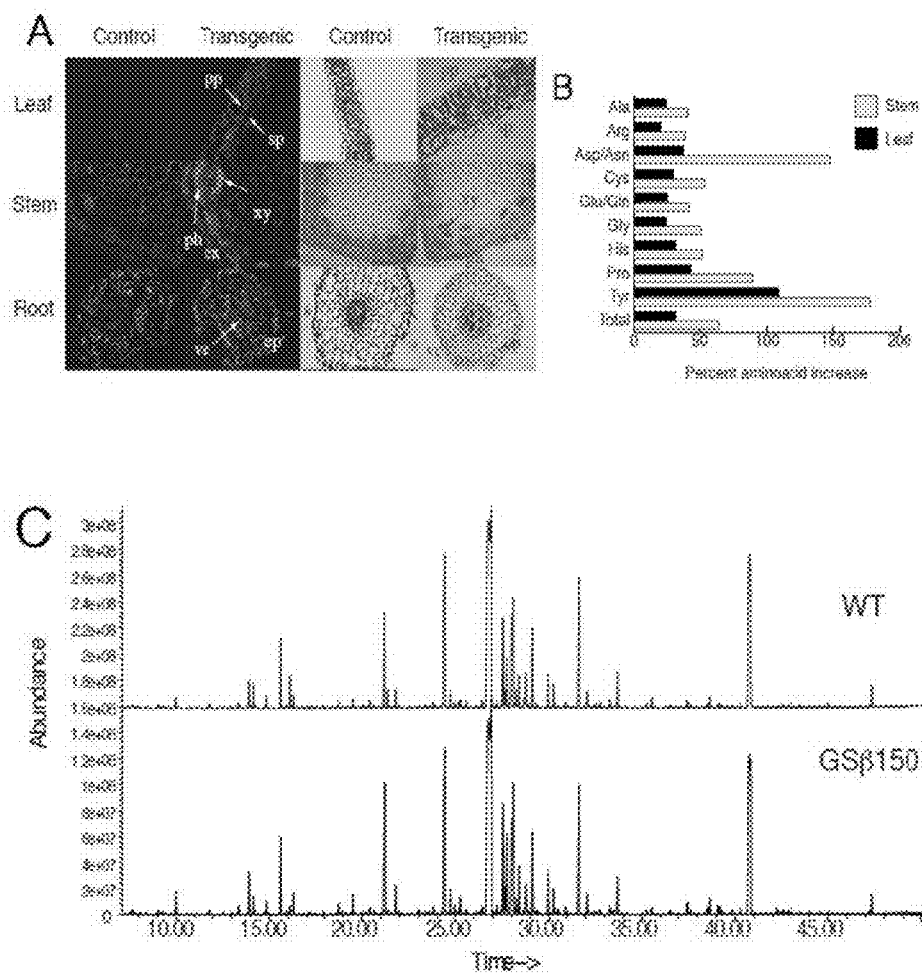
FIG. 28
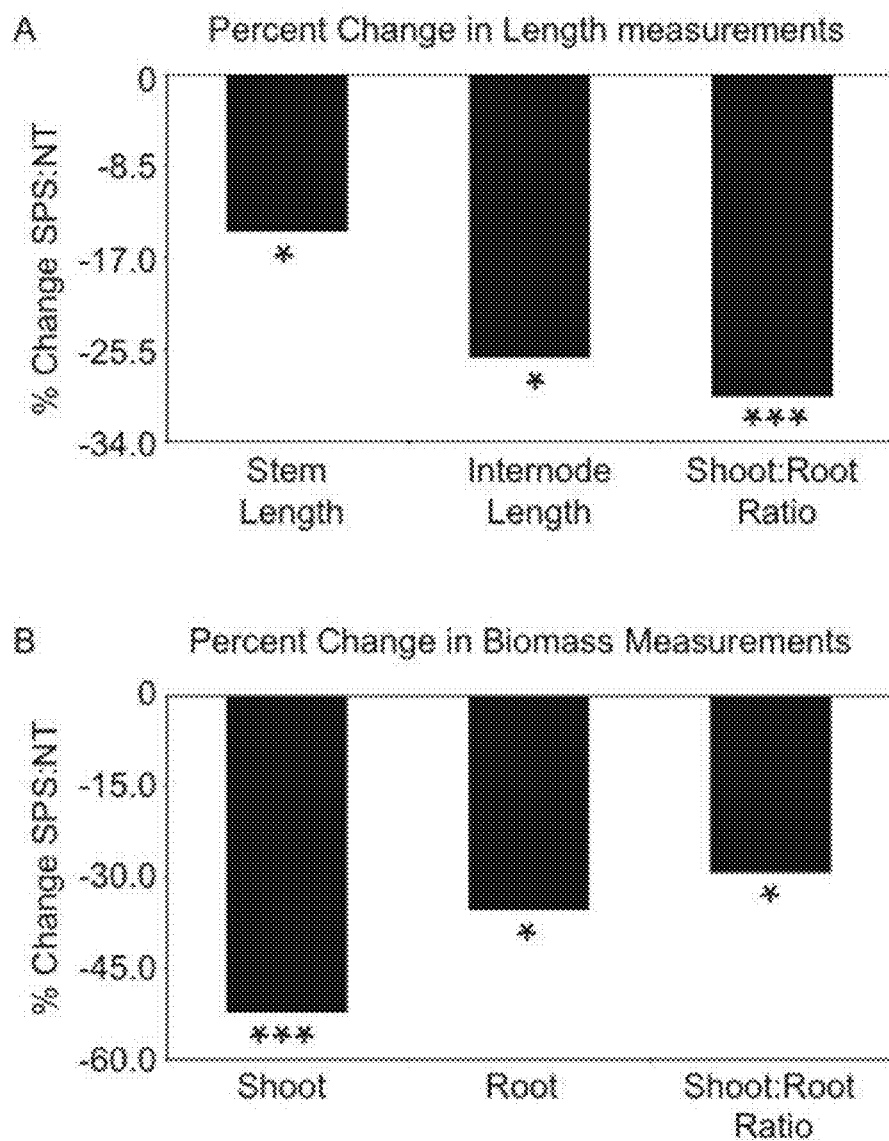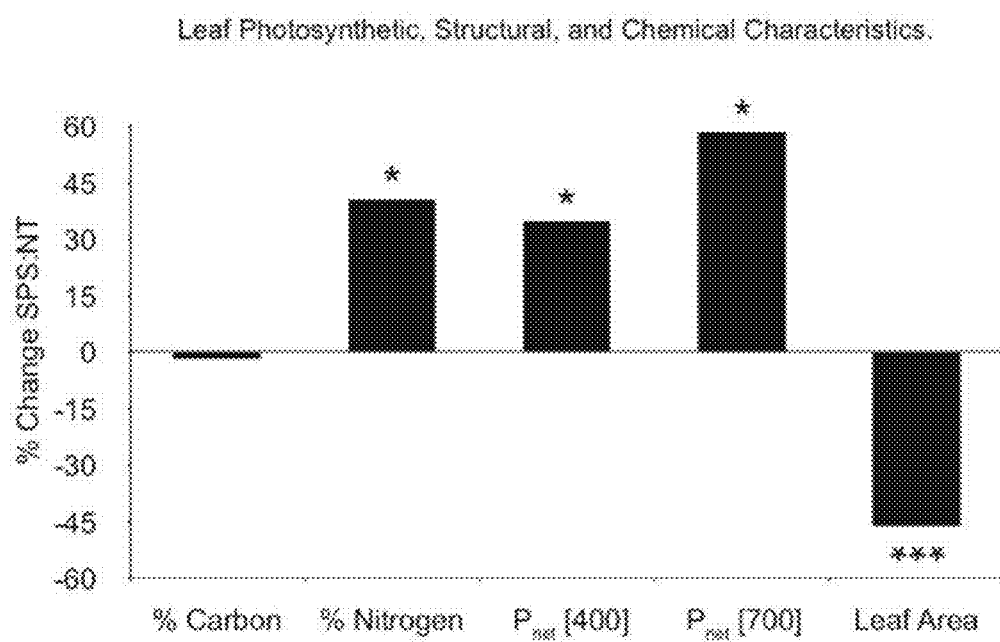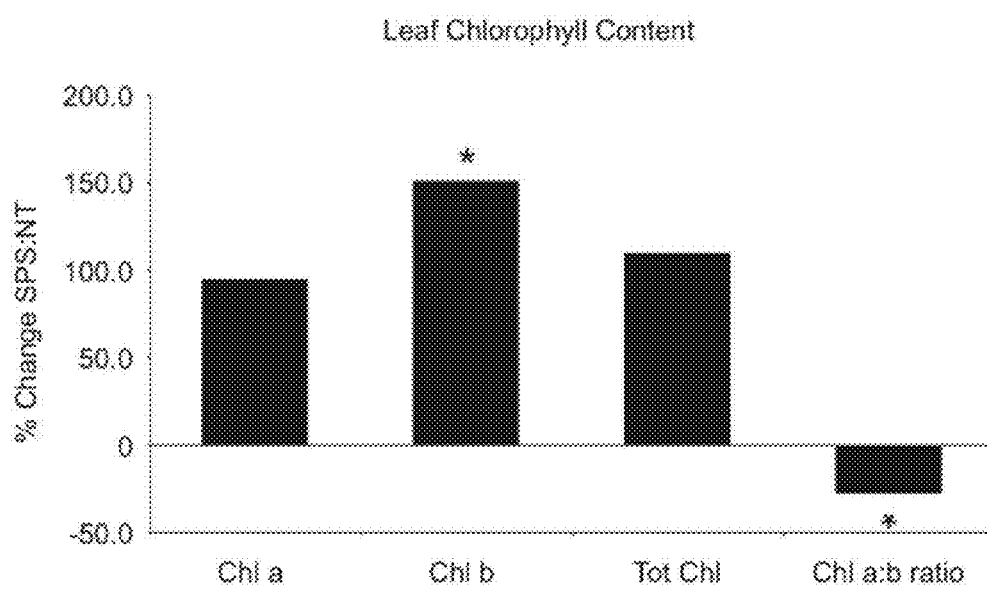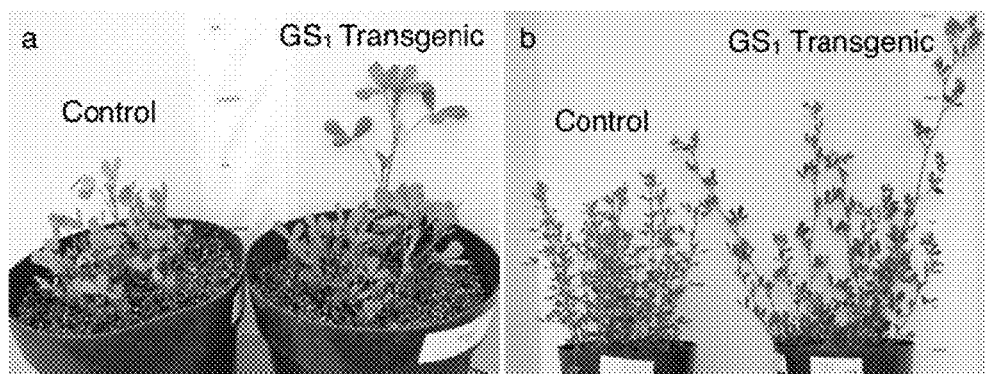
FIG. 29
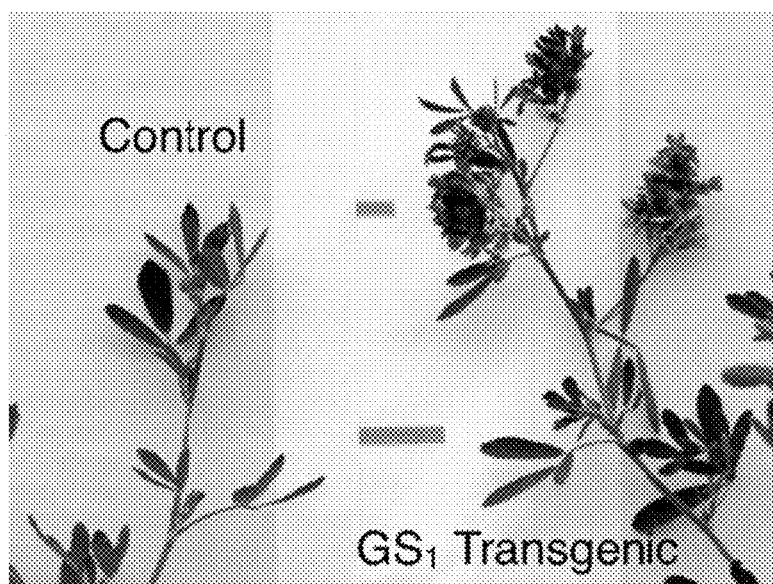
FIG. 30
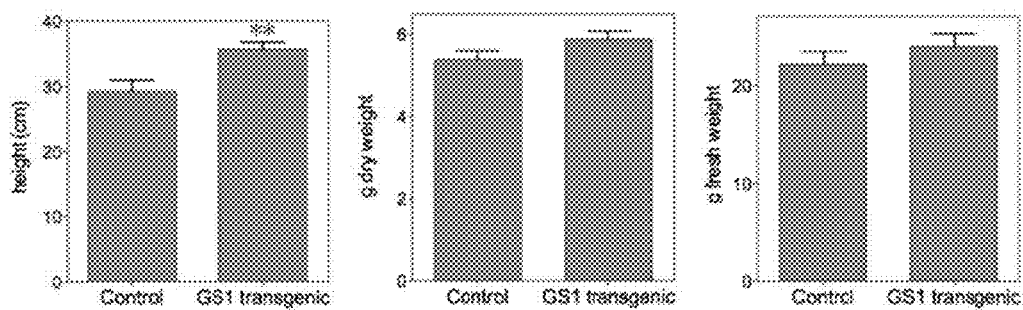
FIG. 31
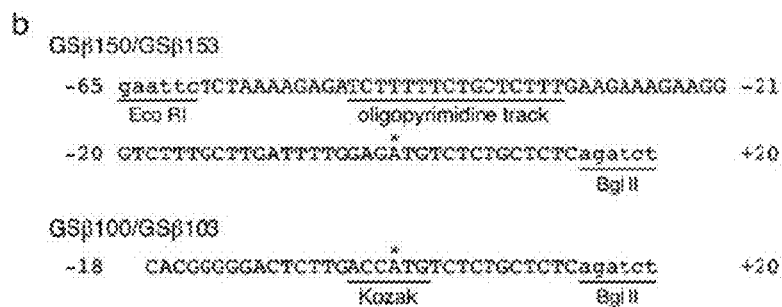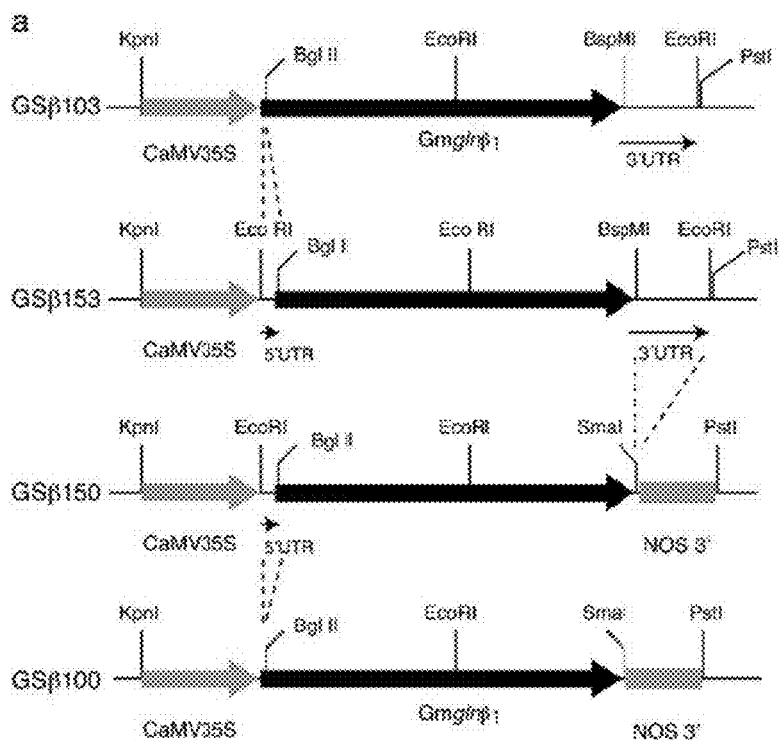
FIG. 32
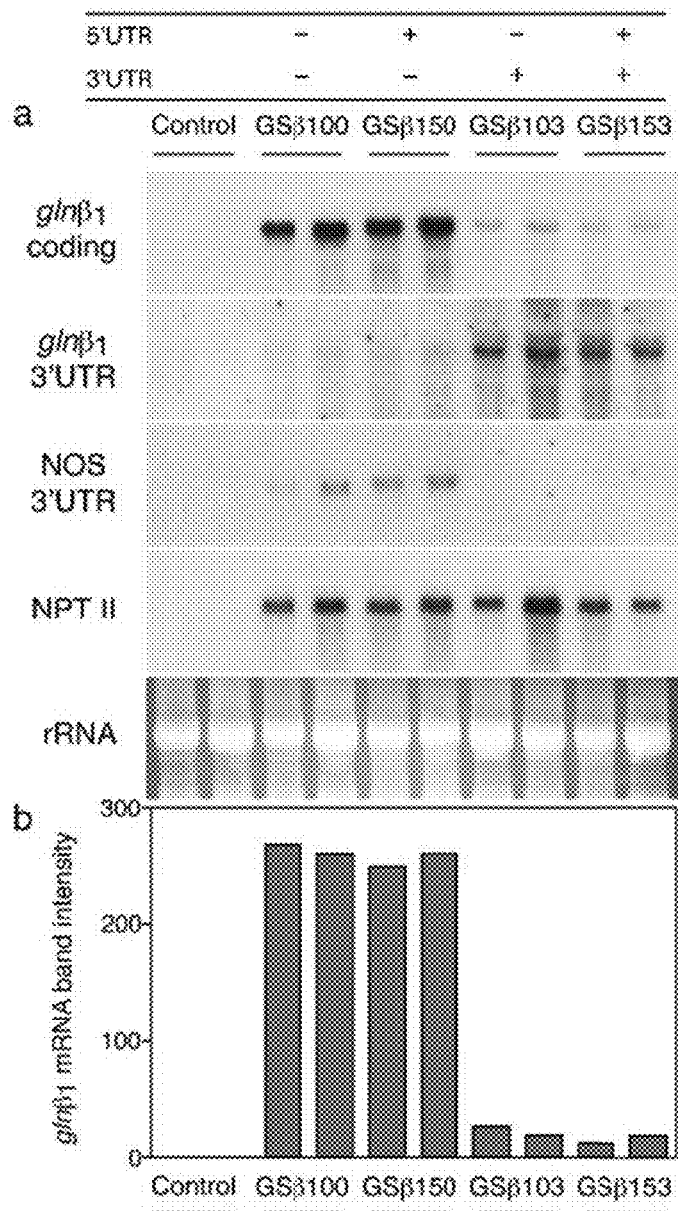
FIG. 33
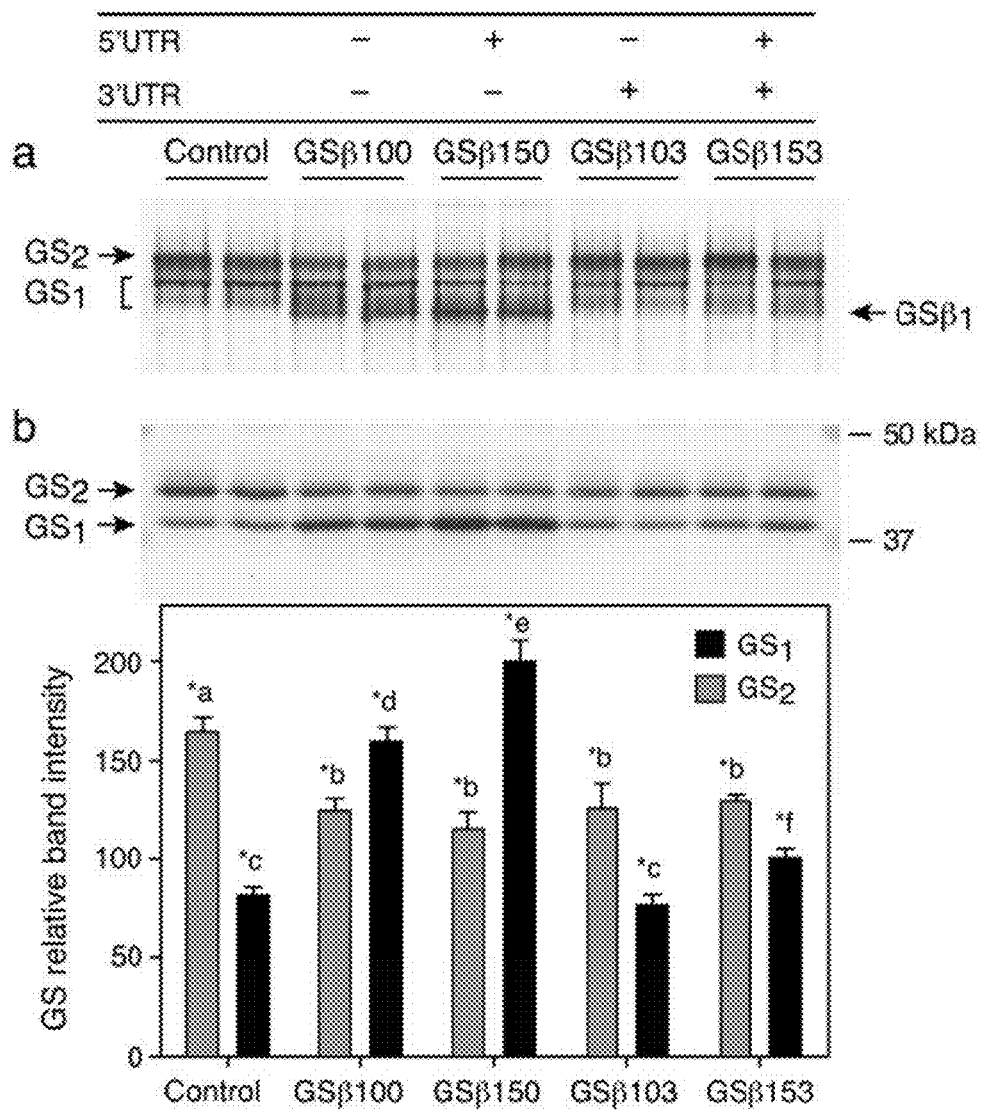
FIG.34
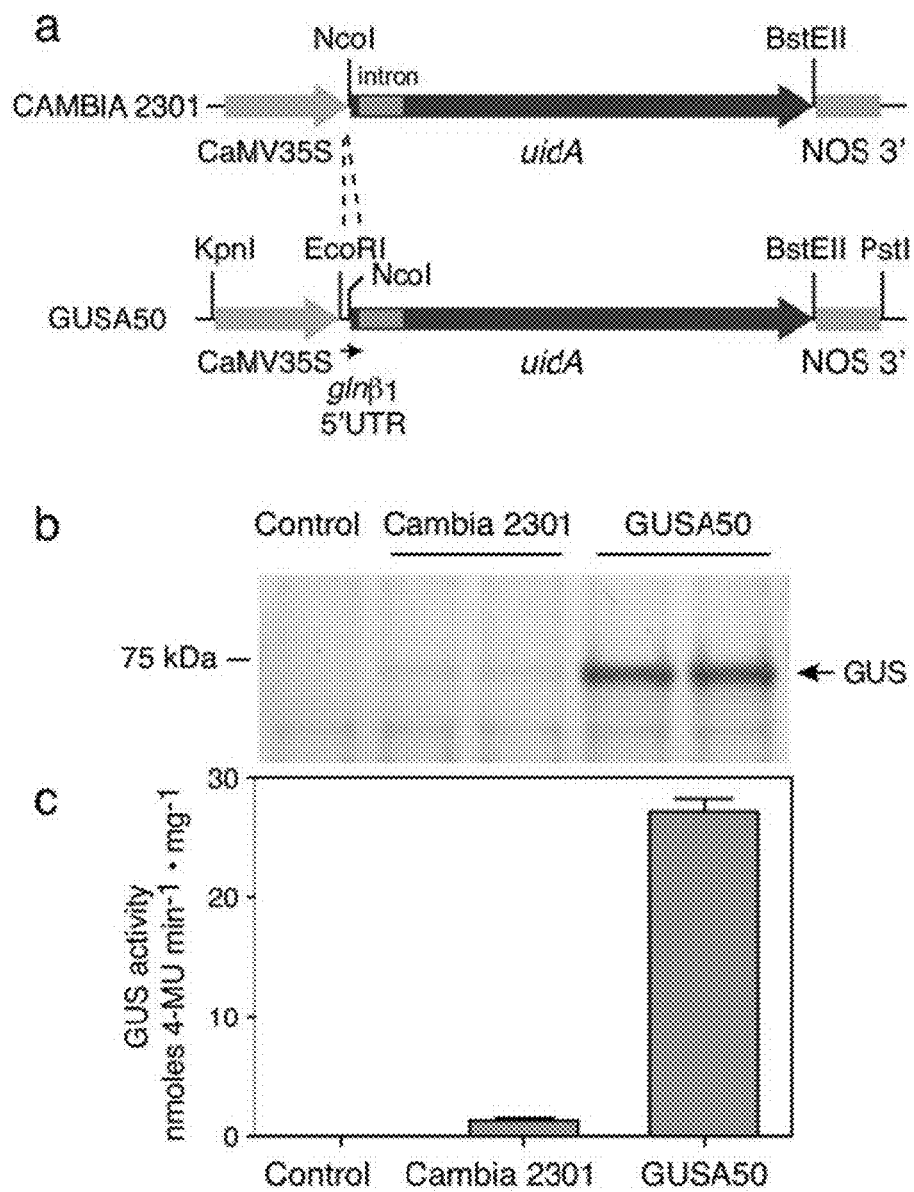
FIG. 35

FIG. 36

SEQ ID NO. 19

FIG. 37

a

pKSglnβ153

Plac 5'UTR   glnβ₁   3'UTR

EcoRI BamHI EcoRI   EcoRI

500 bp

pGEX::glnβ₁

Plac GST 5'UTR glnβ₁ 3'UTR

BamHI EcoRI BamHI EcoRI   EcoRI

b

glnβ₁ 5'UTR

... GST

Gly Ser Pro Gly Ile Leu *
GGATCCCCAGGAATTCTCTAAAAGAGATCTTTTTCTCTCTTT -33
BamHI   EcoRI   Box A

Box A   GSβ₁
                                     Met Ser Leu ...
-32 GAAGAAAGAAGGGTCTTTGCTTGATTTTGGAGATGTCTCTGCT +11
     oligopurine track   SD

SEQ ID NO. 20

c

            Tobacco pKSglnβ135 pKSglnβ15D pGEX4T2   pGEX::glnβ₁
                                                      1   2   3

GS2 →   ~50 kDa
GSβ₁ →   ~37

GST →   ~25 kDa

FIG. 38

TRANSGENIC LEGUMES

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority to and the benefit of the filing of U.S. Provisional Patent Application Ser. No. 61/675, 253, entitled "TRANSGENIC LEGUMES", filed on Jul. 24, 2012, and the specification and claims thereof are incorporated herein by reference.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

This invention was made with government support under USDA grant agreement #2007-35318-18483, and Hatch grants with accession numbers 0198573 and 0197919. The government has certain rights in the invention.

BACKGROUND OF THE INVENTION

1. Field of the Invention (Technical Field)

The present invention relates to the use of the sucrose phosphate synthase (SPS) gene of maize, and closely related regulatory genes, for altering the biosynthesis and accumulation of sucrose in alfalfa and other legumes. The present invention further relates to transgenic constructs containing the SPS and allied regulatory genes, for use in the transformation of alfalfa and other legumes, and it also relates to transgenic plants containing such constructs. The present invention also relates to the use of the glutamine synthetase (GS) gene of soybean, and closely related regulatory genes, for altering the assimilation of nitrogen in alfalfa and other legumes. The present invention further relates to transgenic constructs containing the GS1 gene and allied regulatory genes, for use in the transformation of alfalfa and other legumes, and it also relates to transgenic plants containing such constructs.

2. Description of Related Art

Sucrose phosphate synthase (SPS) is an enzyme used in the synthesis of sucrose in plants. SPS is involved in the synthesis of sucrose by the transfer of glucosyl moiety from UDP-glucose to fructose-6-phosphate, which is dephosphorylated by the action of sucrose-6-phosphate phosphatase (SPP) to yield sucrose. Sucrose is a stable product of photosynthesis that is transported from the photosynthetic tissues via the phloem into heterotrophic tissues which includes the root nodules in leguminous plants. The root nodule is an organ formed as a result of a symbiotic association of legume plants with a soil bacteria, *Rhizobium*. The root nodule is the site where the symbiont can convert free nitrogen into NH_3, which is then assimilated by host encoded enzymes. The symbiont is contained in a membrane bound vesicle called the symbiosome and this membrane is called the peribacteroid membrane which contains many host encoded proteins. The enzyme nitrogenase in the bacteria (symbiont), catalyzes ATP dependent reduction of N_2 to NH_3 in infected cells of the root nodules. Any ammonium produced is exported out through the peribacteroid membrane into the cytosol, where it is assimilated via host encoded enzymes glutamine synthetase (GS) and glutamate synthase to produce glutamine and glutamate.

The nodules are primarily dependent on the import and metabolism of sucrose to fuel the N_2 fixation process. Sucrose is metabolized initially by sucrose synthase (SuSy) (and to a lesser extent vacuolar invertase) and thereafter, via glycolysis to phosphoenolpyruvate (PEP). The products of sucrose

metabolism have two major functions in the nodules. The first is to provide a substrate that can cross the peribacteroid membrane and be oxidized to provide the ATP and reducing power for the fixation of N_2. The second is its involvement in the assimilation of ammonia which is produced by the bacteria, the synthesis of nitrogen products and their export from the nodule via the xylem. The high carbon cost for the alfalfa/ *Rhizobium* interaction renders the nodule a strong sink for sucrose. Furthermore, sucrose is stored in high concentrations in nodules during the photoperiod and utilized in the dark period (at night).

Sucrose plays a role in the nodules. SPS is encoded by a small multigene family. The family members, besides showing differences in tissue-specific expression, are also subject to differential regulation at the posttranslational level via phosphorylation. In alfalfa, SPSB gene shows leaf-specific expression and SPSA gene, though nodule-enhanced, exhibits constitutive expression. Embodiments of the present invention comprise genetically engineered alfalfa that expresses a maize SPSB gene driven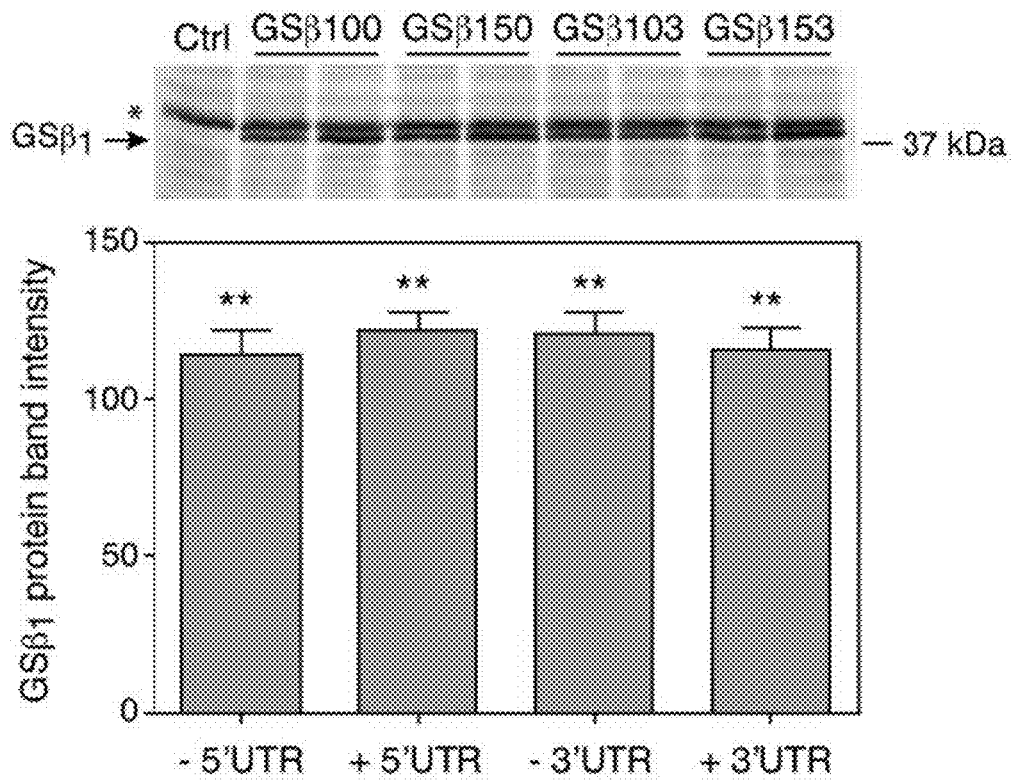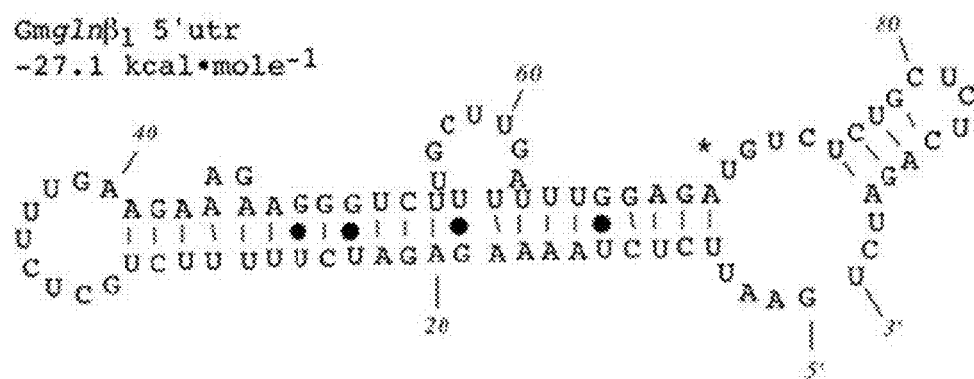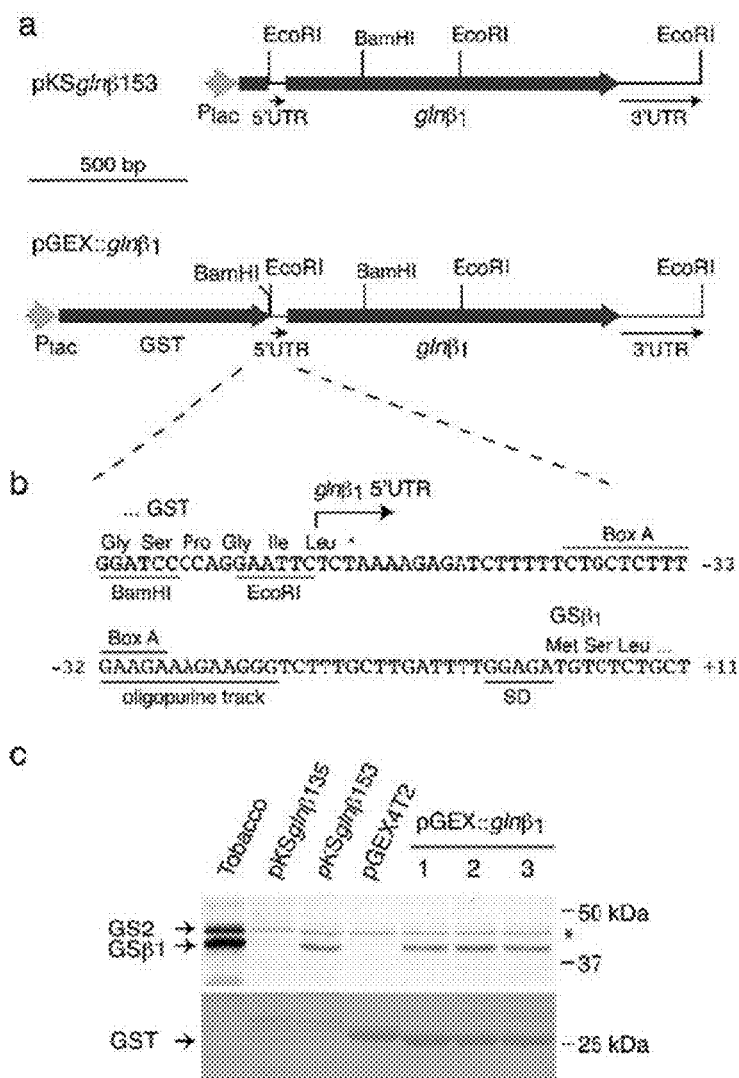 by the CaMV35S promoter. These transformants show increased nodulation and N_2-fixation and overall increased N content at the whole plant level compared to control alfalfa plants. Moreover, the transformants show late flowering, shorter internodes, and intense green coloration mimicking the phenotypes seen in super/hypernodulating legumes.

Glutamine synthetase (GS) plays a central role in nitrogen metabolism in all plants. GS catalyzes the ATP dependent condensation of ammonia with glutamate, to yield glutamine. Plant GS is an octamer and occurs as a number of isoenzyme forms and these GS isoforms are located either in the cytosol (GS1) or chloroplast/plastid (GS2). GS1 represents a key component of nitrogen use efficiency and yield. Increase in GS1 activity is accompanied by a substantial improvement in plant performance.

Regulation of expression of a soybean (*Glycine max*) GS1 gene (Gmglnβ1) driven by a constitutive promoter (CaMV 35S) in transgenic plants typically occurs at a post-transcriptional level. The post-transcriptional regulatory step of the Gmglnβ1 and also the GS1 genes from alfalfa, is at the level of transcript turnover, mediated by their 3'UTR. Gmglnβ1 was investigated to determine if it is also subject to other modes of regulation by testing the role of its 5'UTR in the regulation of gene expression.

The synthesis of glutamine is a first step for the synthesis of all other essential nitrogenous compounds contained in the cells. GS also plays a crucial role in removing ammonia which is toxic to the cells, but at the same time GS levels and activity have to be fine-tuned to maintain a balance between the rates of amino acid biosynthesis and the cellular C skeletons and ATP levels, since they can be depleted by GS activity. GS in bacteria is highly regulated in vivo by transcriptional and post-translational mechanisms including adenylylation and metabolic feedback inhibition. The glutamine/α-ketoglutarate ratio and the adenylate energy charge are critical in the control of GS activity in bacteria. The glutamine/α-ketoglutarate ratio is also important for the control of nitrogen assimilation in plants. GS in plants, as in bacteria, is also regulated at multiple levels.

Analysis of the translation process of eukaryotic mRNAs has shown that the 5'UTR plays an important role in translation initiation by its secondary structure; the context of AUG codon; and the existence of AUG or upstream open-reading frames. The 5'UTR is also the target for the binding of microRNAs that cause translational repression or enhance translation. There are also some reports of the 5'UTR having

a role in mRNA stabilization, though this is usually an attribute of sequences in the 3'UTR.

To demonstrate the role of the 5'UTR of the Gmglnβ1 gene in the regulation of its expression, a series of gene constructs were made with the Gmglnβ1 driven by the CaMV 35S promoter, with and without the 5' and 3' UTRs and tested for their expression by agroinfiltration in tobacco (*Nicotiana tabacum* cv *Xanthi*) leaves, at both the transcript and protein levels. Transient expression through agroinfiltration is a relatively easy procedure known to be effective in analyzing expression of transgenes. Results showed that whereas the 3'UTR of the GS1 gene is involved in the control of the mRNA accumulation or stability, the Gmglnβ1 5'UTR enhances the translation of both the GS1 gene and a β-glucuronidase (uidA) reporter gene in plants.

For a complete characterization of the 5'UTR of the Gmglnβ1 gene and its role as a translation enhancer, the question of whether the 5'UTR of Gmglnβ1 would have the necessary information for allowing initiation of translation in a bacterial cell was also addressed. In the traditional view of translation initiation, there are major differences between eukaryotes and prokaryotes in the way that ribosomes are recruited to the mRNA and this is mediated by sequences in the 5' non-coding region. However, there are reports that eukaryotic ribosomes can recognize prokaryotic signals and initiate synthesis at internal sites of polycistronic mRNAs. Similarly, the *Escherichia coli* ribosomes have been shown to recognize eukaryotic viral initiation signals and translate eukaryotic viral mRNAs suggesting that translation initiation signals in prokaryotes and eukaryotes are similar. To corroborate the universality of the translation initiation mechanism between prokaryotes and eukaryotes, the Gmglnβ1 gene was introduced with its 5'UTR in *E. coli* and showed accumulation of the corresponding protein in the bacterial cells, thus supporting the postulate that the mechanisms of translation initiation for this gene are conserved between plants and bacteria and may further support the notion that GS genes may have originated from a gene duplication event that preceded the divergence of prokaryotes and eukaryotes.

BRIEF SUMMARY OF THE INVENTION

Embodiments of the present invention comprise a transgenic leguminous plant, plant tissue or plant cell comprising an expressible nucleic acid sequence encoding the sucrose phosphate synthase (SPS) gene of maize, said plant, plant tissue or plant cell expressing increased levels of SPS, which catalyzes sucrose synthesis, wherein the nucleic acid comprises a) SEQ ID NO: 1; b) a nucleic acid sequence sharing at least 95% sequence identity with SEQ ID NO: 1; c) an antisense nucleic acid sequence of a) or b); d) a nucleic acid sequence complementary to any one of a) to c); or e) a nucleic acid degeneracy equivalent to any one of a) to d).

Embodiments of the present invention further comprise a transgenic leguminous plant, plant tissue or plant cell comprising an expressible nucleic acid sequence encoding the Glutamine synthetase (GS) gene of soybean, said plant, plant tissue or plant cell expressing increased nitrogen metabolism, wherein the nucleic acid comprises a) SEQ ID NO: 2; b) a nucleic acid sequence sharing at least 95% sequence identity with SEQ ID NO: 2; c) an antisense nucleic acid sequence of a) or b); d) a nucleic acid sequence complementary to any one of a) to c); or e) a nucleic acid degeneracy equivalent to any one of a) to d).

Embodiments of the present invention also comprise a transgenic leguminous plant, plant tissue or plant cell comprising:

I. an expressible nucleic acid sequence encoding the sucrose phosphate synthase (SPS) gene of maize, said plant, plant tissue or plant cell expressing increased levels of SPS, which catalyzes sucrose synthesis, wherein the nucleic acid comprises a) SEQ ID NO: 1; b) a nucleic acid sequence sharing at least 95% sequence identity with SEQ ID NO: 1; c) an antisense nucleic acid sequence of a) or b); d) a nucleic acid sequence complementary to any one of a) to c); or e) a nucleic acid degeneracy equivalent to any one of a) to d); and

II. an expressible nucleic acid sequence encoding the Glutamine synthetase (GS) gene of soybean, said plant, plant tissue or plant cell expressing increased nitrogen metabolism, wherein the nucleic acid comprises a) SEQ ID NO: 2; b) a nucleic acid sequence sharing at least 95% sequence identity with SEQ ID NO: 2; c) an antisense nucleic acid sequence of a) or b); d) a nucleic acid sequence complementary to any one of a) to c); or e) a nucleic acid degeneracy equivalent to any one of a) to d).

Also, embodiments of the present invention comprise the above-mentioned plants wherein the 3' UTR of the GS gene is excluded to enhance GS expression.

Embodiments of the present invention comprise methods to improve the forage quality of a leguminous plant, wherein the method comprise, for example: Transforming a legume plant with a nucleic acid sequence selected from the group consisting of a) a nucleic acid encoding SPS wherein the nucleic acid comprises SEQ ID NO: 1; b) a nucleic acid sequence sharing at least 95% sequence identity with SEQ ID NO: 1; c) an antisense nucleic acid sequence of a) or b); d) a nucleic acid complementary to any one of a) to c); or a nucleic acid degeneracy equivalent to any one of a) to d).; and growing the plant.

Embodiments of the present invention also comprise methods to improve the forage quality of a leguminous plant, wherein the method further comprise transforming the legume plant with a second nucleic acid sequence selected from the group consisting of a) a nucleic acid encoding GS wherein the nucleic acid comprises SEQ ID NO: 2; b) a nucleic acid sequence sharing at least 95% sequence identity with SEQ ID NO: 2; c) an antisense nucleic acid sequence of a) or b); d) a nucleic acid complementary to any one of a) to c); or e) a nucleic acid degeneracy equivalent to any one of a) to d).

Embodiments of the present invention also comprise the above-mentioned methods wherein the 3' UTR of the GS gene is excluded from the second nucleic acid sequence.

Embodiments of the present invention further comprise methods of enhancing translation of foreign genes in plants, plant tissue or plant cells comprising inserting a translation initiation signal from a 5' UTR of a soybean GS gene of SEQ ID NO: 3.

Further scope of applicability of the present invention will be set forth in part in the detailed description to follow, taken in conjunction with the accompanying drawings, and in part will become apparent to those skilled in the art upon examination of the following, or may be learned by practice of the invention. The objects and advantages of the invention may be realized and attained by means of the instrumentalities and combinations particularly pointed out in the appended claims.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

The accompanying drawings, which are incorporated into and form a part of the specification, illustrate one or more

embodiments of the present invention and, together with the description, serve to explain the principles of the invention. The drawings are only for the purpose of illustrating one or more preferred embodiments of the invention and are not to be construed as limiting the invention. In the drawings:

FIG. 1 shows production and identification of putative alfalfa transformed plants constitutively overexpressing the maize SPS gene.

FIG. 1A shows a simple schematic of the 35S-mzSPS construct and several stages in the *A. tumefaciens* mediated plant transformation of alfalfa.

FIG. 1B shows the genomic DNA extracted from the leaves of non-transformed and putative 35S-mzSPS transformed alfalfa plants.

FIG. 2 shows an analysis of maize SPS transcript accumulation in the leaves of 35S-mzSPS transformed plants. Total RNA (about 18 µg) extracted from the leaves of non-transformed (NT; lanes 1-3) and 35S-mzSPS transformed alfalfa (lanes marked 7, 12 and 14) plants was subjected to northern blot hybridization, as described below, using ^{32}P radioactive-labeled cDNA of maize SPS. The gel was stained with SYBR gold and the rRNA bands are shown to represent the loads. A representative blot is shown in Fig. a.

FIG. 3 shows an analysis of Maize SPS polypeptide and holoenzyme accumulation and total SPS activity in the leaves of non-transformed and 35S-mzSPS transformed alfalfa plants.

FIG. 3A shows fifty micrograms of total soluble protein extracted from the leaves of non-transformed (NT) and 35S-mzSPS transformed (7, 12, 14) alfalfa plants, along with maize (mz) leaf extract (about 10 µg), was fractionated by SDS-PAGE (10%) and was subjected to western blot analysis using anti-maize SPS antibodies.

FIG. 3B shows the same leaf extracts (about 100 µg) used in panel 3A, were fractionated by native-PAGE (7.5%) followed by western blot analysis using the same anti-maize SPS antibodies.

FIG. 3C shows the protein extracts used for PAGE in 3A assayed for SPS activity.

FIG. 4 shows a leaf carbohydrate analysis of non-transgenic control and 35S-mzSPS transformed alfalfa plants during the light period, and percent change in the leaf total soluble carbohydrate (TSC), sucrose, and starch content (35-mzSPS/NT) during the light period. Percent change was calculated from data in Table 1 in the appendix. P values were calculated using Students t-test analysis (*, P≤0.05; **, P≤0.01; ***, P≤0.001).

FIG. 5 shows a leaf carbohydrate analysis of non-transgenic control and 35S-mzSPS transformed alfalfa plants during the dark period, and percent change in the leaf total soluble carbohydrate (TSC), sucrose and starch content (35-mzSPS/NT) during the dark period. Percent change was calculated from data in Table 1 in the appendix. P values were calculated using Students t-test analysis (*, P≤0.05; **, P≤0.01; ***, P≤0.01).

FIG. 6 shows an analysis of Maize SPS polypeptide in the leaves of non-transgenic and 35S-mzSPS transgenic alfalfa plants during the light and dark period (day and night).

FIG. 7 shows phenotypic differences observed between the nodulated non-transformed and 35S-mzSPS (#7) transformed alfalfa plants. Images shown are of clonally propagated plants produced via tissue culture that were transferred to the greenhouse and kept under symbiotic N$_2$ fixing conditions.

FIG. 7A shows established non-transformed and 35S-mzSPS (#7) transformed plants were cut back to the base/crown and allowed to grow until the flowering stage.

FIG. 7B shows inflorescence of transformed plants.

FIG. 7C shows side by side comparison of a representative transformed and control plants grown for the same period of time.

FIG. 7D shows inflorescence of control plants at the same developmental stage as the inflorescence from the transformed plants, panel B.

FIG. 7E shows nodulated roots of a representative control plant.

FIG. 7F shows a comparison of the leaf shape between control and transgenic plants. The adaxial side of the first fully expanded trifoliate leaf from the apex of a plant from each set is shown.

FIG. 7G shows nodulated roots of a representative transformed plant. Images represent what was seen in this set of plants and all following sets of plants produced by cuttings.

FIG. 8 shows phenotypic differences in plant height and root size seen between the non-transformed and 35S-mzSPS transformed alfalfa plants. Several sets of cuttings of non-transformed and 35S-mzSPS transformed plants were produced and the root and shoot size and mass were measured.

FIG. 8A shows the data from a representative experiment utilizing three plants from each class grown under symbiotic N$_2$ fixing conditions is shown here. Plants were removed from the pots from two different sets at day 17 (left panel, 8A) and day 30 (right panel 8A) to check for any differences in the roots. The plants seen in the right panel (8A) were used to determine stem:root ratios (maximum length).

FIG. 8B shows a set of plants produced in a hydroponic system using magenta boxes were used to determine stem and root biomass production, as well as for later analysis of leaf and nodule tissue. Picture was taken at 42 days after these plants were established.

FIG. 9 shows percent change in plant growth parameters between N$_2$ fixing non-transformed and 35S-mzSPS transformed alfalfa plants grown under greenhouse conditions.

FIG. 9A shows stem length and internode length was determined from all the stems harvested from non-transformed and 35S-mzSPS transformed plants. Total shoot and root lengths were measured and values were used to calculate shoot:root ratios (length).

FIG. 9B shows shoot and root biomass of non-transformed and 35S-mzSPS transformed alfalfa plants were determined in another set of plants and the values were used to calculate shoot:root ratios (biomass weight). Percent change, calculated from averages in Tables A.5 and A.6, between the transformed and non-transformed plants are shown. (*, P≤0.05; ****, P≤0.001).

FIG. 10 shows percent change in the leaf photosynthetic rates, and changes in total C and N content between non-transformed and 35S-mzSPS transformed plants.

FIG. 11 shows percent change in the leaf chlorophyll content in the non-transformed and 35S-mzSPS transformed plants. Percent change in leaf chlorophyll content calculated from data in Table A.4 of the two sets of plants. P values were calculated using Students t-test analysis (*, P≤0.05).

FIG. 12 shows an analysis of aspartate, asparagine, glutamate, and glutamine in the leaves of the 35S-mzSPS transformed plants.

FIG. 13 shows ethylene production of five non-transformed and 35S-mzSPS transformed alfalfa plants. The estimation of nitrogenase activity in non-transformed (NT) and 35S-mzSPS alfalfa transformed plants was determined via the acetylene reduction assay as described in materials and methods. Average nmoles $C_2H_4.min^{-1}.plant^{-1}$ of five clonally propagated NT and 35S-mzSPS plants ±SE are shown. *P<0.05 with two-tailed Students t-test.

FIG. **14** shows a polypeptide analysis of carbon and nitrogen enzymes in the leaves of non-transformed and 35S-mzSPS transformed plants.

FIG. **15** shows a polypeptide analysis of carbon and nitrogen enzymes in the nodules of non-transformed and 35S-mzSPS transformed plants. Total soluble protein (TSP) extracted from the nodules of non-transformed and 35S-mzSPS transformed alfalfa plants was subjected to SDS-PAGE followed by western blot analysis using antibodies specific for sucrose phosphate synthase (uniSPS; 50 μg TSP) and Maize SPS-specific antibodies (mzSPS; 50 μg TSP)), glutamine synthetase (GS_1; 0.75 μg TSP and GS_2; 7 μg TSP), sucrose synthase (SuSy; 1.5 μg TSP), sucrose phosphate phosphatase (SPP; 3 μg TSP) and phosphoenolpyruvate carboxylase (PePC; 3 μg TSP). Immunoreactive bands and the corresponding enzymes are shown with molecular weight markers (kDa). Coomassie stained gel (15 μg TSP) shown to confirm loads. Band intensities of all the bands were determined, normalized to band intensities obtained from the Coomassie stained gel, and average values ±SE are plotted. $*P<0.05$, $**P<0.01$ with two-tailed Students t-test.

FIG. **16** shows an analysis of SPS and GS_1 polypeptide accumulation in the stem and roots of non-transformed and 35S-mzSPS transformed plants. Total soluble protein (TSP) extracted from the stems and roots of the non-transformed and 35S-mzSPS transformed alfalfa plants was subjected to SDS-PAGE followed by western blot analysis using antibodies specific for glutamine synthetase (GS_1; 1.5 μg TSP) and sucrose synthase (SuSy; 1.5 μg TSP). Immunoreactive bands and corresponding enzymes are shown with molecular weight markers (kDa). Band intensities of all blots were determined and values are shown.

FIG. **17** shows the forage quality of non-transformed and 35S-mzSPS transformed alfalfa plants under symbiotic N_2 fixing conditions.

FIG. **17A** shows protein content/nitrogen profile analysis that was performed on lyophilized shoot tissue. Percent change between the transformed and non-transformed plants of crude protein (CP), soluble protein (SoIP), non-protein nitrogen (NPN), acid detergent insoluble protein (ADIP), and neutral detergent insoluble protein (NDIP) are shown.

FIG. **17B** shows fiber content analysis that was done on the same tissue used in 17A. Acid detergent fiber (ADF) and neutral detergent fiber (NDF) were used to calculate digestible dry matter (DDM) and dry matter intake (DMI), respectively. RFV values are derived from DDM and DMI. Percent change for each parameter was determined using values in Table A.7.

FIG. **18** shows the percent change in plant growth parameters between 5 mM NH_4NO_3 fed non-transformed and 35S-mzSPS transformed alfalfa plants grown under greenhouse conditions. Stem length and internode length was determined from all stems harvested from non-transformed and 35S-mzSPS transformed plants. Total shoot and root lengths were measured and values were used to calculate shoot:root ratios (length). Percent change, calculated from averages in Table A.5, between the transformed and non-transformed plants are shown. ($**$, $P≤0.01$; $***$, $P≤0.001$).

FIG. **19** shows the forage quality of non-transformed and 35S-mzSPS transformed alfalfa plants fed 5 mM NH_4NO_3. Alfalfa non-transformed and 35S-mzSPS transformed plants were cut back to the crown/base and allowed to grow for 40 days at which time shoots were harvested and lyophilized for analysis of forage quality.

FIG. **19A** shows protein (nitrogen) profile of the two sets of plants: Crude protein (CP), soluble protein (SoIP), non-pro-

tein nitrogen (NPN), acid detergent insoluble protein (ADIP), and neutral detergent insoluble protein (NDIP) are shown.

FIG. **19B** shows fiber content analysis was done on the same tissue used in 19A. Acid detergent fiber (ADF) and neutral detergent fiber (NDF) were used to calculate digestible dry matter (DDM) and dry matter intake (DMI), respectively. RFV values are derived from DDM and DMI. Percent change for each parameter was determined using values in Table A.7.

FIG. **20** shows the apical region with new emerging trifoliates of nontransformed and 35S-mzSPS transformed plants over a period of eleven days. A set of non-transformed and 35S-mzSPS transformed plants, transferred to a greenhouse, were immediately inoculated with *Rhizobia* and fed nitrogen free Hoaglands media (FIG. **2A**). Pictures of the apical region were taken every two days to observe and compare the development and expansion of newly formed trifoliate in the 35S-mzSPS transformed plants and control plants. A representative sample of control and transformed plant is shown.

FIG. **21** shows the accumulation of anthocyanin in the stems of all plants and the recovery of pigmentation in the leaves of the transformed plants supplemented with about 5 mM NH_4NO_3.

FIG. **21A** shows stems of non-transformed and 35S-mzSPS transformed plants were bright red in color and indicated an accumulation of Anthocyanin in these tissues. Blue arrows point to the stems of representative plants.

FIG. **21B** shows the set of non-transformed and 35S-mzSPS transformed plants before and after NH_4NO_3 supplementation. Images were taken from trifoliates of representative plants (NT: 1, 2, and 4; SPS: 3, 7 and 14) before NH_4NO_3 supplementation and 29 days after NH_4NO_3 supplementation.

FIG. **22** shows a multistep regulation of Glutamine Synthetase.

FIG. **23** shows a GUS coding region engineered behind a CaMV 35S promoter, followed by different 3'UTRs-3' UTR of Gmglnβ1 (soy), 3'UTR of MsGS100 (GS100), 3' UTR of MsGS13 (GS13), and the NOS terminator (NOS). The constructs were introduced into alfalfa by *A. tumefaciens* mediated transformation. Protein extracts from the leaves of three independent transformants for each construct was subjected to native gel electrophoresis followed by in-gel GUS staining using a flurogenic substrate.

FIG. **24A** shows a Gmglnβ1 gene that was engineered behind a CaMV 35S promoter with and/or without its 3'UTR and/or 5'UTR. The 3'UTR in the GSβ100 and GSβ150 constructs was replaced with a NOS terminator.

FIG. **24B** shows constructs introduced into *A. tumefaciens* strain GV3101. The engineered *A. tumefaciens* were then used for Agroinfiltration into tobacco leaves.

FIG. **24C** shows RNA that was isolated from the infiltrated tissues and subjected to northern blot analysis using the Gmglnβ1 coding region, Gmglnβ1 3'UTR, NOS 3'UTR and the NPTII gene as probes.

FIG. **25A** shows RNA from 53 independent randomly selected transformants with either the GSβ153 construct or the GSβ150 construct were subjected to northern blot analysis using the Gmglnβ1 coding region, Gmglnβ1 3'UTR, NOS 3'UTR and the NPTII gene as probes. The GSβ153 (+) and GSβ150 (−) transformants were identified based on whether the blot hybridized to the Gmglnβ1 3'UTR (+) or the NOS probes (−), respectively.

FIG. **25B** shows the hybridization bands in FIG. **25A**, were scanned and quantified and the band intensity for hybridization with NPTII and the Gmglnβ1 probes was plotted.

FIG. **26**A shows the predicted secondary structure of the 3'UTR of Gmglnβ1 transcript. The secondary structure was predicted using the MFOLD Program. The colored regions indicate the position of conserved AU-rich regions. These sequences in the Amy3 3'UTR are located in domains I and III, which confer sugar-dependent regulation.

FIG. **26**B shows RNA isolated from transformant T13 (exhibiting gene silencing) and transformant T19 (with levels of Gmglnβ1 transcript comparable to NPTII transcript level) was reverse transcribed followed by PCR amplification using oligo(dT) and a gene specific region as primers. The amplified fragments were cloned and sequenced.

FIG. **27** shows leaf RNA and protein from GSβ150 and GSβ153 transformants with equal level of NPTII transcript level. They were subjected to northern blot analysis and native gel electrophoresis followed by western blot analysis, respectively. (A) The RNA blot was hybridized to the Gmglnβ1 coding region, the Gmglnβ1 3'UTR or the NOS probe. (B) Protein extracts from the same transformants as used for RNA analysis was subjected to native gel electrophoresis followed by immunoblot analysis using GS1 antibodies. While the blots showed many immunoreactive bands in all the lanes, a unique band was detected only in the transformants. This band corresponds with the GSM holoprotein. (C) The intensity of the hybridization signal with the Gmglnβ1 coding region probe and the band intensity for same immunoreactive band corresponding to the transgene protein were plotted.

FIG. **28**A shows the site of Gmglnβ1 expression in tissues of GS1β50 alfalfa transformants and the immunolocalization of GS in control and GS1β50 alfalfa transformants. Cross sections of leaves, stem and roots from a non-transformed (control) and the GS1β50 alfalfa transformants (GS) were stained with the GS1 antibodies in combination with a fluorescent antibody. Abbreviations: cx cortex, en endodermis, ep epidermis, pc pericycle, ph phloem, pp palisade parenchyma, sp spongy parenchyma, vc vascular cylinder, xy xylem.

FIG. **28**B shows an increase in total amino acid concentration in alfalfa tissues from transgenic plants compared to the concentration in control non-transformed plants.

FIG. **28**C shows GC-MS total ion chromatograms of leaf extracts from transgenic and wild type alfalfa. The following metabolites with a significant change in accumulation versus wild type plants were identified: Sucrose (28), Inositol (19), Myo Inositol (11), Asparagine (11), Gluconic Acid (10), 2-ketogluconic Acid (10), 2-propenoic Acid (8), Galactose (7), Valine (6), Gluconic Acid (6), Glucose (6), Citric Acid (6), Glutamine (6), Ribonic Acid (6), Fructose (5), Xylitol (3), Proline (3), Benzoic Acid (3), Cystathionine (2), Alanine (2), Cysteine (2).

FIG. **29** shows phenotypic differences between control non-transformed and transgenic alfalfa plants transformed with glutamine synthetase Gmglnβ1 gene (a) two weeks and (b) five weeks of growth after cutting off the shoots.

FIG. **30** shows differences in flowering between control non-transformed and GS1 alfalfa transgenic plants at five weeks of growth after cutting off the shoots.

FIG. **31** shows differences in shoot height and biomass accumulation between transgenic and non-transformed alfalfa plants.

FIG. **32**A shows gene constructs comprising the soybean Gmglnβ1 cDNA driven by the CaMV 35S promoter, either containing or lacking its 5'UTR and/or its 3'UTR.

FIG. **32**B shows the nucleotide sequences of the 5'UTR of the Gmglnβ1 and the CAMBIA leader/Gmglnβ1 gene junctions (GSβ100 and GSβ103 gene constructs). The translation initiation sequence of the CAMBIA leader is underlined.

Nucleotides are numbered relative to the position of the translation start site, labeled with an asterisk. The EcoRI and BglII restriction sites used to clone the Gmglnβ1 gene constructs to the CaMV35S promoter are shown.

FIG. **33** shows transcript analysis of *Agrobacterium* infiltrated tobacco leaf samples.

FIG. **33**A shows the transcript analysis of agroinfiltrated leaf samples.

FIG. **33**B shows that the hybridizing bands corresponding to the Gmglnβ1 coding region and NPTII was quantified and standardized against the NPTII quantified bands and plotted. Two independent experiments were performed and similar results were obtained. Only one set of Northern blots is shown.

FIG. **34** shows western blot analysis of GS protein in leaf extracts of tobacco infiltrated with *A. tumefaciens* containing Gmglnβ1 gene constructs with and without its 5' and 3'UTRs. Proteins were extracted from tobacco leaves 4 days after infiltration with *A. tumefaciens* containing the glnβ1 gene constructs (described in FIG. **32**). For each experiment, two individual leaves were agroinfiltrated with each gene construct. The proteins were fractionated by polyacrylamide gel electrophoresis in either (a) non-denaturing or (b) denaturing conditions (about 10 and 5 μg, respectively), followed by immunoblotting with an antibody against the soybean GSβ1 protein. The experiment was repeated five times and the same results were obtained. The intensities of the GS1 protein bands on the denaturing gel were quantified and the average intensities from five independent experiments ±standard deviation were plotted. A different letter (*a-f) next to a bar means that the differences between these samples were statistically significant (P<0.05). Only one representative Western blot is shown here.

FIG. **35** shows the effect of including the 5'UTR of Gmglnβ1 in gene constructs with uidA reporter gene on the expression of β-glucuronidase (GUS) in agroinfiltrated tobacco leaves.

FIG. **35**A shows tobacco plants were infiltrated with *A. tumefaciens* containing the CAMBIA 2301 plasmid (35SCaMV promoter::uidA reporter gene), or with the GUSA50 gene construct, which comprises the 5'UTR of the Gmglnβ1 gene inserted between the CaMV 35S promoter and the uidA gene of the CAMBIA 2301 plasmid.

FIG. **35**B shows the tobacco plants four days after infiltration protein was extracted from the tobacco leaves and about 50 μg of protein from duplicate samples was subjected to SDS PAGE followed by western blot analysis using anti-GUS antibodies. The control lane is a sample from leaves infiltrated with the CAMBIA 2300 (empty) vector.

FIG. **35**C shows leaf extracts from the uidAinfiltrated leaves that were subjected to GUS enzyme activity determination using the fluorogenic substrate 4-MUG. The molar amount of the 4-methyl umbelliferone (4-MU) produced in the reaction was calculated against a 4-MU standard curve. The experiments were repeated three times with similar results.

FIG. **36** shows analysis of in vitro translation products of in vitro produced transcripts corresponding to the different Gmglnβ1 gene constructs (as described in FIG. **32**). Total RNA was isolated from infiltrated tobacco leaves and reverse transcribed. GS1 PCR products were further amplified from the cDNA with forward primers for the GS1 5'UTR (for constructs GSβ150 and GSβ153) or for the CaMV35S mRNA leader (constructs GSβ300 and GSβ103). RNA was in vitro transcribed from the RT-GSβ1 clones and purified. Equal amounts of capped and polyadenylated RNA were translated in a wheat germ translation system and equal amounts of the

translation reactions were analyzed by western blotting with an anti-GS$_1$ antibody. The position of the GSβ$_1$ immunodetected bands is indicated by an arrow. The presence of an immunoreactive band in the wheat germ extract, that may correspond to the endogenous GS$_1$, is marked with an asterisk. GSβ$_1$ protein bands were quantified and the band intensity values corresponding to the GS constructs with or without its 5'UTR/3'UTR were plotted. The in vitro transcription translation experiments were repeated three times with the same results, only a representative Western blot is shown. (*) Analysis showed that the differences in band intensities due to the presence of either the 5'UTR or the 3'UTR were not statistically significant (P<0.05).

FIG. 37 shows a secondary structure of the 5'UTR of the Gmglnβ$_1$ mRNA. The optimal secondary structure of the 5'UTR, including some nucleotides in the coding region (down to a Bgl II site), was predicted by MFOLD 2.0. The predicted free energy of −27.1 kcal·mole^{-1} was calculated by the UNAFold 3.8 software. The translation start codon is labeled with an asterisk.

FIG. 38 shows an expression of the Gmglnβ$_1$ gene in *Escherichia coli*.

FIG. 38A shows maps of the constructs containing the Gmglnβ$_1$ gene in the pBluescript II KS (pKSglnβ153) or the pGEX-4T-2 (pGEK:glnβ$_1$) plasmids.

FIG. 38B shows the sequence of the GST gene and 5'UTR of the Gmglnβ$_1$ gene junction with regards to sequences that may have a role in translation initiation in prokaryotes. SD represents a Shine-Dalgarno-like sequence.

FIG. 38C shows that the expression of the plasmids comprising the Gmglnβ$_1$ gene in the sense (pKSglnβ153 and pGEX::glnβ$_1$) or the antisense (pKSglnβ135) orientation in *E. coli*, was induced from the P$_{lac}$ or P$_{tac}$ promoters by IPTG. Proteins were extracted after two hours of induction, fractionated by electrophoresis, blotted and immunodetected using the antibodies against the soybean GSβ$_1$ protein, and against the GST protein. A sample from infiltrated tobacco leaves was included as control to indicate the migration pattern of the GSβ$_1$ protein in plants. A control sample from *E. coli* containing the pGEX-4T-2 plasmid was also included for comparison. An asterisk indicates the position of a non-specific antigenic band in *E. coli*. Lane numbers represent samples from three independent single *E. coli* colonies.

DETAILED DESCRIPTION OF THE INVENTION

As used throughout the specification and claims, the term legume or leguminous refers to plants in the family Fabaceae (or Leguminosae), which are notable in that most of them have symbiotic nitrogen-fixing bacteria in their root nodules, including but not limited to alfalfa (lucerne; *Medicago sativa* and *M. falcata* and hybrids between them), white clover (*Trifolium repens*), red clover (*T. pratense*), alsike clover (*T. hybridum*), sweetclover (*Melilotus alba* and *M. officinalis*) and subterranean clover (*P. subterranium*), peas, beans, lentils, lupins, mesquite, carob, soybeans, peanuts, tamarind, and the woody climbing vine *wisteria*. The term alfalfa as used herein is intended to cover all such legumes.

Embodiments of the present invention provide methods for alteration of sucrose synthesis in a variety of plants, such as alfalfa and other forage legumes using the SPS gene. Embodiments of the present invention further provide transgenic constructs comprising the SPS gene, and allied regulatory genes, for use in the transformation of alfalfa and other legumes, and provides novel transgenic plants comprising such constructs.

As used throughout this application, the term sucrose means sugar, or a disaccharide composed of the monosaccharides glucose and fructose with the molecular formula $C_{12}H_{22}O_{11}$. The term carbon can mean the chemical element with symbol C and atomic number 6 but in some instances it can mean organic compounds comprising carbon such as sucrose. Further, as used throughout this application, the term senescence means biological aging or the change in the biology of an organism as it ages after its maturity.

In one embodiment, the SPS gene is used to transform alfalfa for the overexpression of sucrose. The genomic, cDNA and protein sequence of the SPS gene can be obtained in Genbank Accession No. NM_001112224, which provides the following sequence:

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                                        (SEQ. ID. NO: 1)
gaattccggc gtgggcgctg ggctagtgct cccgcagcga

gcgatctgag agaacggtag agttccggcc gggcgcgcgg

gagaggagga gggtcgggcg gggaggatcc gatggccggg

aacgagtgga tcaatgggta cctggaggcg atcctcgaca

gccacacctc gtcgcggggt gccggcggcg gcggcggcgg

gggggacccc aggtcgccga cgaaggcggc gagcccccgc

ggcgcgcaca tgaacttcaa cccctcgcac tacttcgtcg

aggaggtggt caagggcgtc gacgagagcg acctccaccg

gacgtggatc aaggtcgtcg ccacccgcaa cgcccgcgag

cgcagcacca ggctcgagaa catgtgctgg cggatctggc

acctcgcgcg caagaagaag cagctggagc tggagggcat

ccagagaatc tcggcaagaa ggaaggaaca ggagcaggtg

cgtcgtgagg cgacggagga cctggccgag gatctgtcag

aaggcgagaa gggagacacc atcggcgagc ttgcgccggt

tgagacgacc aagaagaagt tccagaggaa cttctctgac

cttaccgtct ggtctgacga caataaggag aagaagcttt

acattgtgct catcagcgtg catggtcttg ttcgtggaga

aaacatggaa ctaggtcgtg attctgatac aggtggccag

gtgaaatatg tggtcgaact tgcaagagcg atgtcaatga

tgcctggagt gtacagggtg gacctcttca ctcgtcaagt

gtcatctcct gacgtggact ggagctacgg tgagccaacc

gagatgttat gcgccggttc caatgatgga gaggggatgg

gtgagagtgg cggagcctac attgtgcgca taccgtgtgg

gccgcgggat aaatacctca agaaggaagc gttgtggcct

tacctccaag agtttgtcga tggagccctt gcgcatatcc

tgaacatgtc caaggctctg ggagagcagg ttggaaatgg

gaggccagta ctgccttacg tgatacatgg gcactatgcc

gatgctggag atgttgctgc tctcctttct ggtgcgctga

atgtgccaat ggtgctcact ggccactcac ttgggaggaa

caagctggaa caactgctga agcaagggcg catgtccaag

gaggagatcg attcgacata caagatcatg aggcgtatcg
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agggtgagga gctggccctg gatgcgtcag agcttgtaat

cacgagcaca aggcaggaga ttgatgagca gtggggattg

tacgatggat ttgatgtcaa gcttgagaaa gtgctgaggg

cacgggcgag gcgcggggtt agctgccatg gtcgttacat

gcctaggatg gtggtgattc ctccgggaat ggatttcagc

aatgttgtag ttcatgaaga cattgatggg gatggtgacg

tcaaagatga tatcgttggt ttggagggtg cctcacccaa

gtcaatgccc ccaatttggg ccgaagtgat gcggttcctg

accaaccctc acaagccgat gatcctggcg ttatcaagac

cagacccgaa gaagaacatc actaccctcg tcaaagcgtt

tggagagtgt cgtccactca gggaacttgc aaaccttact

ctgatcatgg gtaacagaga tgacatcgac gacatgtctg

ctggcaatgc cagtgtcctc accacagttc tgaagctgat

tgacaagtat gatctgtacg gaagcgtggc gttccctaag

catcacaatc aggctgacgt cccggagatc tatcgcctcg

cggccaaaat gaaggggcgtc ttcatcaacc ctgctctcgt

tgagccgttt ggtctcaccc tgatcgaggc tgcggcacac

ggactcccga tagtcgctac caagaatggt ggtccggtcg

acattacaaa tgcattaaac aacggactgc tcgttgaccc

acacgaccag aacgccatcg ctgatgcact gctgaagctt

gtggcagaca agaacctgtg gcaggaatgc cggagaaacg

ggctgcgcaa catccacctc tactcatggc cggagcactg

ccgcacttac ctcaccaggg tggccgggtg ccggttaagg

aacccgaggt ggctgaagga cacaccagca gatgccggag

ccgatgagga ggagttcctg gaggattcca tggacgctca

ggacctgtca ctccgtctgt ccatcgacgg tgagaagagc

tcgctgaaca ctaacgatcc actgtggttc gaccccccagg

atcaagtgca gaagatcatg aacaacatca gcagtcgtc

agcgcttcct ccgtccatgt cctcagtcgc agccgagggc

acaggcagca ccatgaacaa atacccactc ctgcgccggc

gccggcgctt gttcgtcata gctgtggact gctaccagga

cgatggccgt gctagcaaga agatgctgca ggtgatccag

gaagttttca gagcagtccg atcggactcc cagatgttca

agatctcagg gttcacgctg tcgactgcca tgccgttgtc

cgagacactc cagcttctgc agctcggcaa gatcccagcg

accgacttcg acgccctcat ctgtggcagc ggcagcgagg

tgtactatcc tggcacggcg aactgcatgg acgctgaagg

aaagctgcgc ccagatcagg actatctgat gcacatcagc

caccgctggt cccatgacgg cgcgaggcag accatagcga

agctcatggg cgctcaggac ggttcaggcg acgctgtcga
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gcaggacgtg gcgtccagta atgcacactg tgtcgcgttc

ctcatcaaag accccccaaa ggtgaaaacg gtcgatgaga

tgagggagcg gctgaggatg cgtggtctcc gctgccacat

catgtactgc aggaactcga caaggcttca ggttgtccct

ctgctagcat caaggtcaca ggcactcagg tatctttccg

tgcgctgggg cgtatctgtg gggaacatgt atctgatcac

cggggaacat ggcgacaccg atctagagga gatgctatcc

gggctacaca agaccgtgat cgtccgtggc gtcaccgaga

agggttcgga agcactggtg aggagcccag gaagctacaa

gagggacgat gtcgtcccgt ctgagacccc cttggctgcg

tacacgactg gtgagctgaa ggccgacgag atcatgcggg

ctctgaagca agtctccaag acttccagcg gcatgtgaat

ttgatgcttc ttttacattt tgtccttttc ttcactgcta

tataaaataa gttgtgaaca gtaccgcggg tgtgtatata

tatattgcag tgacaaataa aacaggacac tgctaactat

actggtgaat atacgactgt caagattgta tgctaagtac

tccatttctc aatgtatcaa tcggaattc
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The amino acid sequence encoded by the nucleic acid is:

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                                            (SEQ ID. NO. 4)
MAGNEWINGYLEAILDSHTSSRGAGGGGGGGDPRSPTKAASPRGAHMNFNP

SHYFVEEVVKGVDESDLHRTWIKVVATRNARERSTRLENMCWRIWHLARKK

KQLELEGIQRISARRKEQEQVRREATEDLAEDLSEGEKGDTIGELAPVETT

KKKFQRNFSDLTVWSDDNKEKKLYIVLISVHGLVRGENMELGRDSDTGGQV

KYVVELARAMSMMPGVYRVDLFTRQVSSPDVDWSYGEPTEMLCAGSNDGEG

MGESGGAYIVRIPCGPRDKYLKKEALWPYLQEFVDGALAHILNMSKALGEQ

VGNGRPVLPYVIHGHYADAGDVAALLSGALNVPMVLTGHSLGRNKLEQLLK

QGRMSKEEIDSTYKIMRRIEGEELALDASELVITSTRQEIDEQWGLYDGFD

VKLEKVLRARARRGVSCHGRYMPRMVVIPPGMDFSNVVVHEDIDGDGDVKD

DIVGLEGASPKSMPPIWAEVMRFLTNPHKPMILALSRPDPKKNITTLVKAF

GECRPLRELANLTLIMGNRDDIDDMSAGNASVLTTVLKLIDKYDLYGSVAF

PKHHNQADVPEIYRLAAKMKGVFINPALVEPFGLTLIEAAAHGLPIVATKN

GGPVDITNALNNGLLVDPHDQNAIADALLKLVADKNLWQECRRNGLRNIHL

YSWPEHCRTYLTRVAGCRLRNPRWLKDTPADAGADEEEFLEDSMDAQDLSL

RLSIDGEKSSLNTNDPLWFDPQDQVQKIMNNIKQSSALPPSMSSVAAEGTG

STMNKYPLLRRRRRLFVIAVDCYQDDGRASKKMLQVIQEVFRAVRSDSQMF

KISGFTLSTAMPLSETLQLLQLGKIPATDFDALICGSGSEVYYPGTANCMD

AEGKLRPDQDYLMHISHRWSHDGARQTIAKLMGAQDGSGDAVEQDVASSNA

HCVAFLIKDPQKVKTVDEMRERLRMRGLRCHIMYCRNSTRLQVVPLLASRS

QALRYLSVRWGVSVGNMYLITGEHGDTDLEEMLSGLHKTVIVRGVTEKGSE

ALVRSPGSYKRDDVVPSETPLAAYTTGELKADEIMRALKQVSKTSSGM
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One method of the present invention uses the SPS gene sequence in genetic constructs and vectors for transforming plant cells and plant tissues in order to generate transgenic alfalfa plants exhibiting overexpression of sucrose phosphate synthase, which catalyzes sucrose synthesis. Such plants exhibit an increase in leaf to stem ratio, show increased levels of soluble carbohydrates and protein, low fiber, resistance to some biotic and abiotic stresses, delayed senescence, and a significant increase in elemental N, suggesting increased nitrogen use efficiency (NUE).

It is noted that the nucleic acid molecules described in this application represent some of the multiple possible embodiments of the invention. However, the invention also comprises degenerate nucleic acids that differ from the aforementioned sequences. Due to degeneracy in the genetic code, variations in the DNA sequence result in translation of identical peptides. It is thus understood that numerous choices of nucleotides may be made that lead to a sequence capable of directing production of the peptides or functional analogs thereof. Therefore, embodiments of the present invention comprise degenerative nucleotide substitutions.

In accordance with another embodiment of the invention, alterations of the SPS gene can be used, for instance, for expression and functional studies of the encoded protein in alfalfa or other legumes. The DNA sequences can be altered using procedures such as restriction enzyme digestion, DNA polymerase fill-in, exonuclease deletion, terminal deoxynucleotide transferase extension, ligation of synthetic or cloned DNA sequences and site-directed in vitro mutagenesis, including site-directed sequence alteration using specific oligonucleotides together with PCR. The SPS gene sequence can also be altered using site-specific recombination, for instance.

In order to express sucrose phosphate synthase, eukaryotic and prokaryotic expression systems may be generated in which the SPS gene sequence is introduced into a plasmid or other vector, which is then used to transfect living plant cells. Constructs in which the SPS cDNA sequence comprising the entire open reading frame is inserted in a correct orientation into an expression plasmid, can be used for protein expression. Alternatively, portions of the sequence can be inserted. Prokaryotic and eukaryotic expression systems allow various important functional domains of the SPS protein to be recovered as fusion proteins and used for binding, structural and functional studies and also for the generation of appropriate antibodies.

Typical expression vectors comprise promoters that direct the synthesis of large amounts of mRNA corresponding to the gene. As used herein, a suitable promoter may be, for example, but not limited to, a 35S promoter, a nopaline synthase (NOS) promoter, a small subunit rubisco promoter, light-induced promoters, leaf specific promoters, vegetative promoters or any other promoters that are expressed in the desired plant tissue in accordance with the selected application. Plant cells and tissues include, but are not limited to, leaf, stem, flower, root, developing seed, mature seed and seedling. Typical expression vectors can also include sequences allowing autonomous replication within the host organism, sequences that encode genetic traits that allow cells containing the vectors to be selected, and sequences that increase the efficiency with which the mRNA is translated. Stable long-term vectors can be maintained as freely replicating entities by using regulatory elements of viruses. Cell lines can also be produced which have integrated the vector into the genomic DNA and in this manner the gene product is produced on a continuous basis.

Another embodiment of the invention comprises a transgenic plant or plant cell transformed with a nucleic acid comprising a nucleotide sequence encoding the amino acid sequence of SPS and wherein the nucleotide sequence is expressed. In one embodiment, the plant or plant cell is alfalfa, which is transformed with and expresses a nucleic acid comprising the nucleotide sequence of SPS. The plant or plant cell can be a monocot or a dicot. Preferably, the plant or plant cell is a legume, for example, alfalfa. The invention further provides a method for overexpressing sucrose in a legume plant that comprises, but is not limited to: Transforming a legume plant with a nucleic acid sequence from, including but not limited to:

a) a nucleic acid encoding a coding region of the SPS regulatory gene;

b) a nucleic acid coding for a SPS regulatory gene sequence;

c) a nucleic acid sequence sharing at least 85% sequence identity with a) or b);

d) a nucleic acid degeneracy equivalent to any one of a) to c); or

f) a nucleic acid fragment exhibiting SPS gene biological activity of any one of a) to d); and growing the plant.

According to a further embodiment of the invention, the SPS gene can be used in a plant, plant cell or tissue, to increase sucrose content for improving forage quality and as a result, improving animal health, carcass weight gain, milk and wool production and decreasing bloat in animals. Such plants include, but are not limited to, alfalfa (lucerne; *Medicago sativa* and *M. falcata* and hybrids between them), white clover (*Trifolium repens*), red clover (*T. pratense*), alsike clover (*T. hybridum*), sweetclover (*Melilotus alba* and *M. officinalis*) and subterranean clover (*P. subterranium*).

In accordance with a further aspect of the present invention, a genetic construct, comprising the SPS nucleotide sequence, can be used under the control of a suitable promoter that is capable of expression in a designated plant part, e.g. the leaf. The genetic construct preferably transforms alfalfa and other forage legumes, increases tolerance or resistance to infection by fungi, viruses and/or bacteria; and, increases tolerance or resistance to disease, insects, nematodes, and other pest species.

There is a high degree of constraints in overexpressing GS1. Alfalfa transformants with a soybean GS1 gene (Gmglnβ1) driven by the CaMV 35S promoter previously showed no increase in GS1 polypeptide or GS activity. For example, there was transgene transcript accumulation in the leaves but not in the nodules. Furthermore, the alfalfa transformants showed a significant drop in the level of the transcript corresponding to the transgene when grown under nitrogen sufficient conditions. Since the CaMV 35S promoter is active in the nodules and its activity is not influenced by nitrate, it can be concluded that the expression of GS1 is regulated at the level of transcript stability. The absence of an increase in GS1 polypeptide or activity in the leaves where there is transgene transcript accumulation, indicates that there is regulation also at a translational or post-translational level. Embodiments of the present invention comprise a method of removing 3' UTR from the GS1 transgene (Gmglnβ1). The removal of 3' UTR from the GS1 transgene abolishes the regulatory constraints in the expression of the transgene in alfalfa and the transformants show not only increased GS1 transcript, but also an increase in the GS1 polypeptide and a novel GS isoenzyme. Moreover, results from the method show that the 3'UTR mediated posttranscriptional regulation requires a product of N assimilation.

GS1 encoded by Gmglnβ1 is regulated at the level of transcript turnover and/or translation initiation and this regulatory step is mediated via the 3'UTR. Furthermore, a trans-factor or micro RNA (miRNA) is involved in transcript turnover/translation repression. The trans-factor is either synthesized de novo or an existing protein is posttranslationally modified in response to the C/N ratio in the cells. If miRNA is involved, it can be synthesized under certain metabolic conditions associated with NH3 assimilation and it can target the GS1 mRNA for turnover and/or translation repression.

In one embodiment, the GS1 gene is used to transform alfalfa plants. The genomic, cDNA and protein sequence of the GS1 gene can be obtained in Genbank Accession No. AF301590.1, which provides the following sequence:

```
                             (SEQ. ID. NO: 2)
tctaaaagag atcttttct gctctttgaa gaaagaaggg

tctttgcttg attttggaga tgtctctgct ctcagatctc

atcaaccttta acctctccga taccaccgag aaggtgatcg

cagagtacat atggatcggt ggatcaggaa tggacctgag

gagcaaagca aggactctcc caggaccagt tagcgaccct

tcagagcttc ccaagtggaa ctatgatggt tccagcacag

gtcaagctcc tggtgaagac agtgaagtga ttttataccc

acaagccatt ttcagggatc cattcagaag gggtaacaat

atcttggtta tctgtgatgc ctacactcct gctggagaac

ctattcccac taacaagagg cacgctgctg ccaaggtttt

cagccatcct gatgttgttg ctgaagtgcc atggtacggt

attgaacaag aatacacctt gttgcagaaa gatatccaat

ggcctcttgg gtggcctgtt ggtggtttcc ctggacctca

gggtccatac tactgtggtg ttggcgctga caaggctttt

ggccgtgaca ttgttgacgc acactacaaa gcctgtattt

atgctggcat caacatcagt ggaattaatg gagaagtgat

gcccggtcag tgggaattcc aagttggacc ttcagttgga

atctcagctg gtgatgagat ttgggcagct cgttacatct

tggagaggat cactgagatt gctggtgtgg tggtttcctt

tgaccccaag ccaattaagg gtgattggaa tggtgctggt

gctcacacaa actacagcac caagtccatg agagaagatg

gtggctatga agtgatcaaa gcagcaattg acaagttggg

gaagaagcac aaggagcaca ttgctgctta tggagaaggc

aacgaacgtc gtttgacagg acgccacgaa accgctgaca

tcaacacctt cttatgggga gttgcaaacc gtggagcttc

tgttagggtt gggagagaca cagagaaagc agggaaggga

tattttgagg acagaaggcc agcttccaac atggacccat

acgtggttac ttccatgatt gcagacacaa ccattctgtg

gaagccatga gcaaaacctg catgttttct ccctttggat

ggaaaggaac agttatgctt ttcttagtag gatttggtct
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ctctctcttt ttaccttttg attggtacta tggttggtgc

cttgttggtt ggtgcaacta actggcaagg gttgttcatt

gttttcttct attcctttcc ctcgtttttcc gattgttaca

atgacaataa tttaatggtt attatcagtc ttgaacaaag

aaatgctgat tgtgaagtat aataataata tatgaaattg ccg
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The amino acid sequence encoded by the nucleic acid is:

```
                                    (SEQ ID. NO. 5)
MSLLSDLINLNLSDTTEKVIAEYIWIGGSGMDLRSKARTLPGPVSDPSELP

KWNYDGSSTGQAPGEDSEVILYPQAIFRDPFRRGNNILVICDAYTPAGEPI

PTNKRHAAAKVFSHPDVVAEVPWYGIEQEYTLLQKDIQWPLGWPVGGFPGP

QGPYYCGVGADKAFGRDIVDAHYKACIYAGINISGINGEVMPGQWEFQVGP

SVGISAGDEIWAARYILERITEIAGVVVSFDPKPIKGDWNGAGAHTNYSTK

SMREDGGYEVIKAAIDKLGKKHKEHIAAYGEGNERRLTGRHETADINTFLW

GVANRGASVRVGRDTEKAGKGYFEDRRPASNMDPYVVTSMIADTTILWKP
```

While most of the genetic manipulations have utilized one gene at a time and used a run-of-the-mill constitutive CaMV 35S promoter, an embodiment of the present invention fine-tunes the expression such that the transgenes are expressed in an organ/tissue-specific manner. In a different embodiment of the invention, introduction of both the transgenes into the same plant is performed behind different regulatory elements, such that the SPS gene, is expressed in the leaves and the GS$_1$ in the stems, roots and nodules. The dual gene transformants preferably enhance the traits that are with the individual gene transformants.

In one embodiment of the invention, the 5' UTR of the Gmglnβ$_1$ gene is used as a translation enhancer for foreign genes in plants. Specifically, the cDNA sequence of the 5' UTR of the Gmglnβ1 gene being as follows:

```
                                     (SEQ ID NO: 3)
tctaaaagag atcttttct gctctttgaa gaaagaaggg

tctttgcttg attttggag
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INDUSTRIAL APPLICABILITY

The invention is further illustrated by the following non-limiting examples.

Example 1

A maize SPS gene (mzSPS) cDNA was engineered between the constitutive CaMV35S promoter and the NOS terminator in the vector pMON316. The construct referred to as the 35S-mzSPS was introduced into alfalfa using a routine *A. tumefaciens* mediated transformation protocol and putative 35S-mzSPS alfalfa transformants were selected based on their ability to grow and root on selection media containing kanamycin. These putative alfalfa transformants, along with non-transformed (NT) alfalfa plants were transferred to pots and grown in the greenhouse. DNA was isolated from the leaves of these transformants and subjected to PCR amplification using NPTII gene specific primer sets. The PCR products were then analyzed by agarose gel electrophoresis.

Referring to FIG. 1A, a schematic of the 35S-mzSPS construct and several stages in the *A. tumefaciens* mediated plant transformation of alfalfa are shown beginning with infected leaf tissue (1), followed by cell proliferation/expansion (2), and the formation of calli (3), embryos (4) and plantlets (5). Fully developed plants (6) were placed and grown in pots under greenhouse conditions and then used for further analysis. Referring to FIG. 1B, genomic DNA extracted from the leaves of non-transformed and putative 35S-mzSPS transformed alfalfa plants was subjected to PCR using NPT II, kanamycin resistance gene, specific primers. An Invitrogen® 1 kb plus DNA ladder (lane 1), empty vector (+control; lane 2) and samples from both the non-transformed (NT) and putative transformants (35S-mzSPS) were tested. The specific 800 bp amplified fragment obtained with the positive control template and the transformants is indicated in the gel shown. While nine randomly selected transformants used in this experiment along with the positive control (plasmid DNA) showed the presence of a 800 bp fragment, the NT plants and the negative control (*A. tumefaciens* with the empty vector) did not show the band. These results confirmed the integration of the tDNA with the 35S-mzSPS gene construct along with the NPTII gene in the transformants.

To check for the functionality of the transgene construct, total RNA extracted from the leaves of three randomly picked 35S-mzSPS transformants and three NT plants were subjected to northern blot analysis using the mzSPS coding region as a probe. As seen in FIG. 2, under hybridization conditions, the probe did not hybridize to the RNA from the NT plants but showed a distinct hybridizing band in the transformants representing the mzSPS transcript. The mzSPS transcript level showed plant to plant variation, with the highest level being in transformant #7. The results indicate that the gene construct is functional with regards to transcription and the differences in the expression level is probably an attribute of position effect.

To check if mzSPS transcript accumulation in the leaves of the transformants translates into a functional protein, the same tissues that were used for RNA extraction, were used for SPS protein and SPS enzyme activity analysis. Total soluble protein extracted from the leaves was subjected to SDS- and native-PAGE western blot analysis using maize SPS antibodies. As seen in FIG. 3A, both the NT plants and the 35S-mzSPS transformants showed an immunoreactive band of about 140 kD that co-migrated with an immunoreactive band in the lane with the maize leaf extract on SDS PAGE, the level being about half of that seen in the maize leaf extract.

Considering that the amount of leaf extract loaded for maize leaf was 5-fold less than for the leaf extracts of alfalfa, it would seem that the alfalfa transformants showed about 10-fold less SPS than the maize leaf. The band in the NT plant extract was many fold less intense than the band in the extracts from the transformants. The immunoreactive band in the samples from NT plants probably represents the endogenous SPS protein which has some affinity for the maize SPS antibodies.

Since SPS is a dimeric protein in its native form, the transgene protein was checked to see if it was assembling into a dimer by performing native gel western analysis with the leaf extracts. As seen in FIG. 3B, the immunoreactive band corresponding to the maize SPS polypeptide migrated at about 140 kDa (3A) and the maize SPS holoenzyme migrated at about 280 kDa (3B), as shown in the lane marked mz, which serves as the positive control. The migration of the molecular weight standards is shown.

SPS activity was assayed in the same leaf protein extracts used for western analysis (panel 3A and 3B). Average values ±SE of three experiments were plotted as nmol Sucrose-P min-1 mg-1 native gel western analysis showed a broad immunoreactive band only in the maize leaf extract and in the extracts from the three transformants. The band intensity of native SPS protein showed a correlation with the mzSPS transcript level. The absence of an immunoreactive native protein in the NT plants likely reflects the low affinity of the alfalfa SPS native protein for the mzSPS antibody or just a reflection of the relatively low levels of the holoprotein in the control plants compared to the transformants.

SPS enzyme is known to undergo post-translational modification that can affect its activity and as such, it is critical that the SPS transgene product is active in its non-native environment. The same protein extracts used for PAGE were assayed for SPS activity. SPS enzyme activity was measured by the quantification of fructosyl moiety of sucrose using the anthrone test in triplicate for each plant and as seen in FIG. 3C, the transformants showed higher SPS activity compared to the non-transformed plants. An approximately 50-120% increase in total SPS activity was seen in the leaves of the three 35S-mzSPS transformed plants, where transgenic line 35S-mzSPS #14 showed the highest levels, followed by #7 and #12. Taken together, these results indicate that the expression of the 35S-mzSPS gene in alfalfa is accompanied by an increase in SPS activity in the leaves. Transformant #7 was selected for further analysis.

The pathways of sucrose and starch formation are interdependent as they compete for the pool of triose phosphates produced by the Calvin cycle. A consequence of this complex interdependence is that sucrose formation and starch formation are reciprocally related. Thus, increasing SPS activity could have an impact on the sucrose and starch levels when the leaves are in the photosynthetic mode. To check this postulate, total soluble carbohydrate (TSC), sucrose, and starch content was determined in the leaves of NT alfalfa and 35S-mzSPS transformed plants during the light and dark period. A set of 10 clonally propagated NT alfalfa and 35S-mzSPS (#7) transformed plants were placed under greenhouse conditions and inoculated with *S. meliloti*. Leaf tissue from five NT and five transformed alfalfa plants were harvested at midday representing the light period and another set of plants were harvested at midnight representing the dark period on that same day for carbohydrate analysis.

Referring to FIG. 4, during the light period, the transformed plants exhibited an approximately 39 and 64 percent increase in leaf TSC and sucrose content (P<0.001), respectively, when compared to the non-transformed plants.

TABLE A.1

Total soluble carbohydrate (TSC), sucrose, and starch content in the leaves of
alfalfa non-transformed and 35S-mzSPS transformed plants during the light period.

NT	T.S.C (μmol g FW-1)	Sucrose (μmol g FW^{-1})	Starch (μmol g FW^{-1})	T.S.C.: Starch ratio	Sucrose: Starch ratio
2a	25.1	8.0	477.3	0.053	0.017
2b	24.5	7.0	500.0	0.049	0.014

TABLE A.1-continued

Total soluble carbohydrate (TSC), sucrose, and starch content in the leaves of alfalfa non-transformed and 35S-mzSPS transformed plants during the light period.

NT	T.S.C (μmol g FW-1)	Sucrose (μmol g FW^{-1})	Starch (μmol g FW^{-1})	T.S.C.: Starch ratio	Sucrose: Starch ratio
2c	25.1	8.0	ND	ND	ND
2d	27.2	9.0	701.2	0.039	0.013
2e	24.2	7.0	ND	ND	ND
Average	25.2	7.8	559.5	0.047	0.015
S.E.	±0.2	±0.2	±24.6	±0.001	±0.000
35S-mzSPS					
7a	32.0	10.0	ND	ND	ND
7b	40.4	14.0	521.8	0.077	0.027
7c	31.4	14.0	ND	ND	ND
7d	34.0	12.0	315.8	0.108	0.038
7e	37.4	14.0	542.8	0.069	0.026
Average	35.0	12.8	460.1	0.085	0.030
S.E.	±0.8	±0.4	±25.1	±0.004	±0.001
% change	38.9	64.1	−17.8	80.9	107.9
t-test	0.0006	0.0005	0.3831	0.0386	0.0184

In Table A.1 above, leaf tissue from five non-transformed and five 35S-mzSPS transformed alfalfa plants were analyzed for total soluble carbohydrate, sucrose and starch levels. Values for each plant are shown along with TSC to starch ratios and sucrose to starch ratios. ND represents data that was not determined. Percent change was calculated from averages and P values were calculated using a two-sided Student t-test.

In FIG. **4**, the transformed plants were approximately 18 percent lower in leaf starch content than the non-transformed plants (P=0.3831) during the same period. Consequently, the calculated TSC:starch and sucrose:starch ratios were approximately 80 and 110 percent higher, respectively, in the transformed plants when compared to the NT plants (P<0.05).

Referring now to FIG. **5**, the leaf TSC in the transformed plants showed an about 32% increase over NT plants during the dark period (P<0.001; see table A.2 below).

TABLE A.2

Total soluble carbohydrate (TSC), sucrose, and starch content in the leaves of alfalfa non-transformed and 35S-mzSPS transformed plants during the dark period.

NT	T.S.C. (μmol g FW-1)	Sucrose (μmol g FW^{-1})	Starch (μmol g FW^{-1})	T.S.C.: Starch ratio	Sucrose: Starch ratio
2a	20.5	4.0	ND	ND	ND
2b	20.5	4.0	499.4	0.041	0.008
2c	22.7	5.0	371.2	0.061	0.013
2d	21.3	5.0	702.9	0.030	0.007
2e	23.0	6.0	ND	ND	ND
Average	21.6	4.8	524.5	0.044	0.010
S.E.	±0.2	±0.2	±33.5	±0.003	±0.001
35S-mzSPS					
7a	27.6	6.0	ND	ND	ND
7b	26.9	5.0	ND	ND	ND
7c	29.6	6.0	727.5	0.041	0.008
7d	30.6	8.0	615.6	0.050	0.013
7e	27.5	6.0	795.6	0.035	0.008
Average	28.4	6.2	712.9	0.042	0.010
S.E.	±0.3	±0.2	±18.2	±0.002	±0.001
% change	31.6	29.2	35.9	−5.7	0.7
t-test	0.0001	0.0528	0.1617	0.8152	0.9818

Percent change in carbohydrates from the light period to dark period:

	T.S.C.	Sucrose	Starch	TSC:Starch	Sucrose:Starch
NT	−14.3	−38.5	−6.2	−5.6	−34.4
35S-mzSPS	−18.8	−51.6	54.9	−50.8	−68.2
NT-t-test	0.001	0.000	0.785	0.806	0.096
SPS-t-test	0.007	0.000	0.047	0.027	0.008

Table A.2 shows the total soluble carbohydrate (TSC), sucrose, and starch content in the leaves of non-transformed and 35S-mzSPS transformed alfalfa plants during the dark period. Leaf tissue from five non-transformed and five 35S-mzSPS transformed alfalfa plants were analyzed for total soluble carbohydrate, sucrose and starch levels. Values for each plant are shown along with TSC to starch ratios and sucrose to starch ratios. ND represents data that was not determined. Percent change was calculated from averages between the two sets (NT and SPS) during the dark period and P values were obtained by using a two-sided Student t test. Same calculations were done comparing data from the light period to the dark period within each class of plants.

As shown in FIG. 5, while the leaf sucrose and starch content was also substantially higher, approximately 30 and 35 percent (respectively), in the transformed plants during the same period, these differences were not significant (P=0.053 and P=0.16, respectively). As such, no significant changes were seen in the calculated TSC:starch and sucrose:starch ratios when comparing the two classes of plants (P=0.81 and p=0.98, respectively; FIG. 5 and Table A.2) indicating C partitioning was not as enhanced to sucrose biosynthesis in the transformants. Hence, the increased SPS activity seen in the leaves of the transformed alfalfa plants enhanced carbon partitioning to sucrose synthesis only during the light period at which time the leaves are photosynthetically active.

Furthermore, the absence of photosynthetic activity in the leaves during the dark period resulted in a significant reduction in leaf TSC and sucrose content in both classes of plants compared to the levels seen in the light period (Table A.2). However, the starch content in the leaves of the transformed plants during the dark period was significantly higher than the levels during the light period (P<0.05; Table A.2). As such, the calculated leaf TSC:starch and sucrose:starch ratios showed a significant drop in the transformed plants (P<0.05 and P<0.01, respectively; Table A.2). On the contrary, the NT plants showed no significant changes in sucrose:starch ratios between the two time periods. The NT plants analyzed during the dark period showed approximately a 6 percent drop in starch content when compared to the plants analyzed during the light period.

SPS in some plants is known to be light regulated and the regulation may be by protein phosphorylation, allosterically by Glc-6-P and Pi, and in the case of developing leaves also by changes in the protein level. To check if changes in the sucrose level in light and dark period could be attributed to changes in SPS levels, total soluble protein (TSP), extracted from the leaves of the same plants analyzed during the two time periods, was subjected to SDS-PAGE western blot analysis using the maize SPS antibody. About 10 μg of maize leaf protein used in FIG. 6, was used as a positive internal control in both gels.

Referring to FIG. 6, fifty micrograms of total soluble protein extracted from the leaves of non-transformed (NT) and 35-mzSPS alfalfa transformed plants during the light 6A and dark period 6B, along with maize (mz) leaf extract (about 10 μg), was fractionated by SDS-PAGE (10%) and was subjected to western blot analysis using anti-maize SPS antibodies. The maize leaf extract was used as control to account for differences in blotting conditions between the two gels. The blots from a representative experiment are presented. Arrows indicate the immunoreactive band corresponding to maize SPS polypeptide and the stained Rubisco polypeptide that migrate at approximately 140 kDa and 40 kDa, respectively. The migration of the molecular weight standards is as shown. The maize protein showed two major immunoreactive bands: about 140 kD band and a about 90 kD band, the latter probably representing a breakdown product of the 140 kD SPS protein. Both, the transformants and the NT plants showed higher accumulation of the SPS protein (140 kD and 90 kD immunoreactive bands) in the tissues harvested in the light period compared to the tissues harvested in the dark period.

The change in maize SPS polypeptide accumulation in the leaves of the transformants followed a similar trend as seen in the sucrose content during the two periods with the higher accumulation of SPS protein seen during the light period. The data, shows the diurnal/circadian regulation pattern of the maize SPS at the protein level in alfalfa.

Clearly, the heterologous expression of the maize SPS transgene in alfalfa results in enhanced carbon partitioning to sucrose synthesis in the leaves and an increase in the sucrose: starch ratios during the light period and this is attributed to the diurnal changes in SPS level.

Increased SPS activity attained by introducing SPS transgene in different plants is associated with different phenotypes like increased fruiting and freeze tolerance, greater biomass in tomato, and a higher rate of cellulose synthesis. The transformant #7 along with a NT alfalfa plant was propagated via regeneration in tissue culture. The regenerants were moved into a vermiculite and sand mixture in pots in the greenhouse and were inoculated with S. meliloti. Under the symbiotic conditions, the transformants showed striking differences in the phenotype (FIG. 7). One of the more pronounced differences seen immediately between the two sets of plants was in leaf pigmentation. The leaves of the transformed plants displayed a darker shade of green (Bluish green) when compared to the NT plants (FIG. 7F). Leaf morphology was also altered in the transformed plants with the leaflets of each trifoliate being smaller in size and narrower in width (FIG. 7F). As these plants were allowed to grow, a clear effect on growth/height (FIGS. 7A and 7C, FIG. 8) was seen in the transformed plants along with a delay in the emergence of flowers. Only 12 percent of the 35S-mzSPS (#7) transformed plants showed signs of flowering approximately 10-14 days after 100 percent of the non-transgenic plants flowered (FIGS. 7B and 7D), about 8 weeks after being cut back. While the 35S-mzSPS transformed plants that showed signs of flowering had two to three flowers per inflorescence, the number of these also being very low at one to two per plant, the NT alfalfa plants had approximately 15-20 inflorescence per plant with each inflorescence containing approximately 10 flowers (FIGS. 7B and 7D). Additionally, the root system of the transformed plants had many more nodules, many of which formed large pronounced clusters compared to the non-transformed plants, which had a much smaller number of nodules, smaller size and in smaller clusters (FIGS. 7E and 7G). These trends were observed consistently in three consecutive sets of clonally propagated NT and 35S-mzSPS transformed plants produced via cuttings and when all plants were allowed to grow after being cut back to the crown/base.

Shoot to root ratios, stem length, and internode length per stem were determined in both sets of plants to quantitatively evaluate the differences seen in plant growth at the phenotypic level. Twenty clonally propagated NT and 35S-mzSPS (#7) transformed alfalfa plants, produced via cuttings, were established in the greenhouse and inoculated with S. meliloti. Approximately 30 days post inoculation (FIG. 8A, right panel and 8B), total shoot and root length was measured in each plant to calculate shoot to root ratios (length). While no significant difference was seen in root length between the two sets of plants, the stem length was approximately 22 percent lower in the 35S-mzSPS transformed (#7) plants (data not shown; P<0.01). As seen in FIG. 9A, shoot to root ratios (length) derived from the above values were approximately 30 percent lower in the transformed plants compared to the NT plants (P<0.01). Moreover, shoot and root biomass (wet weight) was approximately 52 and 35 percent lower, respectively, in the transformed plants (FIG. 9B and Table A.6).

TABLE A.6

	Shoot Biomass			Root Biomass			Shoot:Root Ratio		
Shoot and root biomass measurements of alfalfa non-transformed and 35-mzSPS transformed plants grown under symbiotic N, fixing conditions.

Samples	Shoot Biomass			Root Biomass			Shoot:Root Ratio		
N2 Fixing	Average	S.E	t-test	Average	S.E.	t-test	Average	S.E.	t-test
NT	4.06	±0.13	0.000	4.12	±0.19	0.034	1.05	±0.04	0.046
SPS	1.94	±0.07		2.87	±0.11		0.74	±0.02	
% Change	−52.22			−35.19			−29.52		

The values obtained from these measurements were used to calculate shoot:root biomass ratios (wet weight) and the transformed plants were significantly lower when compared to the non-transformed plants (FIG. 9B). The experiment was repeated and similar results were obtained. This data demonstrated a negative effect of the transgene on plant growth in the 35S-mzSPS transformed plants when kept under symbiotic N_2 fixing conditions. However, the reduction in the length of the internodes of the transformed plants represented an increase in the leaf to stem ratios, which is indicative of enhanced forage quality. The phenotype exhibited by transformant #7 was also displayed by all other independent transformants with the 35S-mzSPS gene that were analyzed (data not shown), confirming the phenotype to be a result of increased SPS activity.

Referring to FIG. 10, percent change in leaf carbon and nitrogen content (% carbon and % nitrogen, respectively), net photosynthetic rates under ambient (P_{net} [400]) and elevated (P_{net} [700]) CO_2 conditions, and leaf area between non-transformed and 35S-mzSPS transformed plants was determined from data in Table A.3 below.

mately 35 percent under ambient CO_2, compared to the P_{net} values taken under elevated CO_2 conditions (data not shown). While the NT plants showed an approximately 14 percent increase in photosynthetic rates when comparing P_{net} values of the two CO_2 conditions, this increase was not significant (P=0.38). Furthermore, the average leaf area of each trifoliate measured for P_{net}, was approximately 46 percent lower in the transformed plants when compared to the NT plants (FIG. 10 and Table A.3). Thus, the 35S-mzSPS transformed plants exhibited higher photosynthetic rates under both CO_2 conditions, albeit the smaller leaf size, and responded better to elevated CO_2 conditions as shown by the higher increase in P_{net} values when comparing P_{net} under the two CO_2 conditions.

Moreover, the difference in pigmentation in the leaves of the transformed plants (FIG. 8) suggest a change in the nitrogen status of the plants. As such, chlorophyll content and elemental composition of carbon and nitrogen were deter-

TABLE A.3

Leaf photosynthetic, structural, and chemical characteristics of non-transformed and 35S-mzSPS transformed plants.

	% Carbon		% Nitrogen		Amax 400 umol		Amax 700 umol		Leaf area	
Sample	Average	S.E.	Average	S.E.	Average	S.E.	Average	S.E.	Average	S.E.
NT	41.4	±0.3	3.3	±0.2	15.8	±0.48	18.1	±0.8	2.6	±0.06
35S-mzSPS	40.7	±0.5	4.7	±0.1	21.29	±0.31	28.6	±0.7	1.4	±0.01
	−1.7		42.4		34.7		58.0		−46.2	
% change	0.68		0.04		0.01		0.01		0.00	
t-Test										

P values were calculated using Students t-test analysis (*, P≤0.05; ***, P≤0.001). Photosynthetic rates in the 35S-mzSPS transformed plants were approximately 35 and 58 percent (P≤0.05) higher under ambient and elevated CO_2 conditions, respectively, when compared to the NT plants. Moreover, the 35S-mzSPS transgenic plants showed a higher increase (P≤0.05) in photosynthetic rates (P_{net}), approxi-

mined in the leaves of the NT and 35S-mzSPS transgenic alfalfa plants to address any changes in these parameters. As seen in FIG. 11 and Table A.4 below, pigment analysis of the 35S-mzSPS transformed plants showed approximately a 100 and 150 percent increase in the levels of chlorophyll a and chlorophyll b, respectively, when compared to the NT plants.

TABLE A.4

Leaf chlorophyll content of alfalfa non-transformed and 35S-mzSPS transformed plants.

	Chl a		Chl b		Tot Chl		Chl a:b ratio	
Sample	Average	S.E.	Average	S.E.	Average	S.E.	Average	S.E.
NT	481.9	±28.8	175.1	±15.3	656.92	±43.9	2.44	±0.11
35S-mzSPS	941.0	±97.5	439.5	±43.9	1380.44	±140.4	1.77	±0.06
% change	95.3		151.4		110.1		−27.5	
t-Test	0.078		0.034		0.059		0.024	

These values were combined and a similar trend was seen with the transformed plants exhibiting approximately 110 percent higher increase in total chlorophyll content. Statistical analysis of the three data sets showed significant changes in the levels of chlorophyll b (FIG. 11; Table A.4; P≤0.05). Elemental analysis of the leaves showed no significant difference in carbon content between the two sets of plants (FIG. 10; Table A.3; P=0.68). Conversely, nitrogen content was approximately 42 percent higher in the transformed plants when compared to the NT plants (P≤0.05). These results indicate the 35S-mzSPS transgenic plants, under symbiotic N_2-fixing conditions, have more available nitrogen than NT plants as represented by the higher nitrogen and chlorophyll content seen in the leaves. This would further imply that the increased N level is due to enhanced nodulation and the accompanying increase in N_2-fixation.

To determine any changes in N_2 fixation rates, acetylene-reduction by nitrogenase was assayed using the entire root system of NT and transformed alfalfa plants. As seen in FIG. 13, the nodules of the transformed plants exhibited approximately 44 percent increase in the acetylene-reduction rates (ethylene production; C_2H_4 nmoles.min-1.plant-1) when compared to the NT plants. Therefore, the transformed plants showed enhanced N_2 fixation rates when compared to the NT plants.

Referring to FIG. 14, total soluble protein, extracted from the leaves of the two classes of plants, was subjected to SDS-PAGE western blot analysis using antibodies specific to key enzymes in carbon and nitrogen metabolism. As previously seen in FIG. 3, significant accumulation of the maize SPS polypeptides was seen in the leaves of the transformed plants. The use of the universal and maize SPS antibodies showed a similar increase in band intensity of approximately 64 and 81 percent, respectively, in the immunoreactive bands corresponding to SPS in the protein extract of the transformed plants when compared to the extract from NT plants (FIG. 3). Total soluble protein (TSP) extracted from the leaves of the same non-transformed and 35S-mzSPS transformed alfalfa plants used in FIG. 6, was subjected to SDS-PAGE followed by western blot analysis using antibodies specific for sucrose phosphate synthase (uniSPS; 50 µg TSP) and Maize SPS-specific (mzSPS; 50 µg TSP)), glutamine synthetase (GS_1; 7 µg TSP and GS_2; 7 µg TSP), sucrose synthase (SuSy; 3 µg TSP), sucrose phosphate phosphatase (SPP; 3 µg TSP) and phosphoenolpyruvate carboxylase (PePC; 3 µg TSP). Immunoreactive bands and corresponding enzymes are shown with molecular weight markers (kDa). Coomassie stained gel (15 µg TSP) is shown to confirm protein loads. Band intensities of all bands were determined, normalized to band intensities obtained from the Coomassie stained gel, and average values ±SE were plotted. *P<0.05, **P<0.01 with two-tailed Students t-test. While the antibodies showed affinity for the endogenous SPS, the higher level of SPS polypeptide accumulation seen in the transformed plants compared to NT plants, can be attributed to the maize SPS transgene protein. While no significant differences were seen in the accumulation of GS, Susy, or SPP polypeptides in the leaves of the transformed plants, a significant increase was seen in band intensities that corresponded to the immunoreactive bands of PePC (FIG. 14).

Up-regulation of SPS activity has been shown to increase sink number and enhance sink strength in several plant species. The root nodules of legumes are a unique C sink in that they are metabolically highly active in fixing free N and assimilating the fixed nitrogen. Higher N content in the transformed plants compared to NT plants when grown under N_2-fixing conditions, suggests higher levels of N_2-fixation

and assimilation in the nodules of the transformants (FIGS. 10, 11, and 12). In FIG. 12, amino acids were extracted from non-transformed and transformed alfalfa plants. Samples, along with standards for Asp, Asn, Glu, and Gln, were analyzed via liquid chromatography-mass spectroscopy (LCMS) to determine any change in the levels of these amino acids in the leaves of the transformed plants compared to control plants. Average values in $pmol·mg$ $FW^{-1}±S.E.$ bars are shown. Moreover, higher N metabolic activity in the nodules would imply higher import of sucrose from the leaves. As discussed earlier, the nitrogen fixation rates were higher in the transformants compared to the NT plants (FIG. 13).

To check if increased SPS activity in the photosynthetic tissues affects import of sucrose into the nodules which could eventually affect the expression of other genes encoding enzymes with roles in C and N metabolism, the levels of the several enzymes were checked in the nodules. Total soluble protein, extracted from the nodules of the two classes of plants, was subjected to SDS-PAGE western blot analysis using antibodies specific to key enzymes in carbon and nitrogen metabolism. A significant increase in the accumulation of SPS polypeptides was seen in the nodules of the transformed plants. As seen in FIG. 14, the presence of endogenous SPS in the nodules was confirmed via the use of the universal SPS antibody. The immunoreactive bands corresponding to SPS polypeptides (about 140 kDa) were of lower intensity in the protein extract from the NT plants when compared to the extract from the transformed plants (about 2.8 fold; P<0.05). The higher band intensity seen in the nodules of the transformed plants represents the accumulation of both the endogenous SPS and maize SPS polypeptides. A more pronounced difference between the two sets of plants in SPS polypeptide accumulation was seen with the use of the maize specific antibody.

However, no difference was seen between the two sets of plants in the band intensities of sucrose-6-phosphate phosphatase (SPP, about 40 and 50 kDa), an enzyme also involved in the synthesis of sucrose, along with SPS. Accumulation of the enzyme sucrose synthase (SuSy, about 90 kDa), known for its central role in modulating sink strength and maintaining proper nodule function was higher in the transformed plants, as shown by the average band intensities of the immunoreactive bands corresponding to SuSy (P<0.05, FIG. 14). Interestingly, no difference was exhibited between the two classes of plants in the accumulation of phosphoenolpyruvate carboxylase (about 110 kDa; P>0.05,), which also plays an important role in the nodule by recycling carbon via anaplerotic CO_2 fixation. On the contrary, the nodules of the transformed plants exhibited a significant increase in the intensities of immunoreactive bands corresponding to cytosolic (GS_1, about 39 kDa) and plastidic (GS_2, about 42 kDa) glutamine synthetase of approximately 92 and 74 percent, respectively. While accumulation of PePC polypeptides showed no difference between the two classes of plants, the changes in GS_1 and GS_2 polypeptide accumulation is indicative of a response of these enzymes to the increased rates of N_2 fixation seen in the transformed plants (FIG. 15).

In addition to the nodules, other C sinks such as the roots and stems may be affected by the constitutive expression of maize SPS in alfalfa. To address this, SuSy and GS polypeptide accumulation was analyzed in TSP, extracted from the stems and roots of NT, and transformed plants to determine any changes in sink strength or ammonium assimilatory capabilities in these organs. As seen in FIG. 16, the accumulation of GS protein was similar between the two classes of plants in both sets of tissues. However, a substantial increase was seen in SuSy polypeptide accumulation in the roots of the trans-

formed plants and there was in general an increase also in the stem. Nonetheless, the 35S-mzSPS transformed plants clearly exhibited enhanced sink strength in heterotrophic tissues as seen by the higher level of SuSy protein in these tissues. Moreover, these plants showed improved N_2-fixing capabilities and the higher levels of GS polypeptide in the nodules are indicative of enhanced NH_4^+ assimilatory capabilities.

The leaves of forage crops are of higher quality than stems and the previous data indicate the 35S-mzSPS transformed plants have a higher leaf:stem ratio when compared to the NT plants (FIGS. 8 and 9). Moreover, the transformed plants showed higher nitrogen and chlorophyll content in the leaves as well as a delay in flowering time which implies a delay in senescence (FIGS. 10 and 11). Hence, a complete nitrogen profile and fiber analysis was conducted on the two sets of plants to determine if there were any changes in forage quality in the transformed plants.

Referring to FIG. 17, shoots of twenty clonally propagated NT and 35S-mzSPS transformed plants, kept under symbiotic N_2-fixing conditions, were harvested 40 days after being cut back to the crown/base and freeze dried via lyophilization. Data from pooled tissue is presented as percent of dry matter with the exception of soluble protein (SoIP) which is presented as percent of crude protein (CP). As seen in FIG. 17 and Table A.7, CP percentages in the shoots of the transformed plants were approximately 33 percent higher than the NT plants.

gen) bound to fiber was derived by subtracting the values of ADIP from NDIP of which, the 35S-mzSPS transformed plants demonstrated approximately 68 percent higher level of degradable/digestible protein (nitrogen) bound to fiber than the NT plants (data not shown).

Furthermore, neutral detergent fiber (NDF) and acid detergent fiber (ADF) analysis was performed on the same tissue from both sets of plants to evaluate fiber content. The NDF values, representing total cell wall components was approximately 25 percent lower in the 35S-mzSPS transformants when compared to the NT plants. Similarly, the most indigestible cell wall component (such as lignin), represented as ADF values showed an approximate drop of 20 percent in the 35S-mzSPS transformed plants. Dry matter digestibility (DDM) and dry matter intake (DDI) of the two sets of plants were determined using the ADF and NDF values, respectively. Estimations of DDM and DDI were further used to calculate the relative feed value (RFV), an index and ranking forages by potential digestible dry matter intake. As seen in FIG. 17B, more or less similar values of DDM were seen in both sets of plants with increase in the 35S-mzSPS transformed plants compared to the NT plants. However, a substantial increase in DDI and the calculated RFV content was seen in the transformed plants of approximately 33 and 39 percent, respectively.

Taken together, the 35S-mzSPS alfalfa transformed plants showed a substantial increase in the level of readily available protein (CP, SoIP, and NPN) and degradable/digestible pro-

TABLE A.7

Forage quality and value of shoots harvested from alfalfa non-transformed and 35S-mzSPS transformed plants grown under different nitrogen conditions.

Sample N₂ Fixing	CP (% DM)	SoIP (% CP)	NPN (% DM)	NDIP (% DM)	ADIP (% DM)	NDF (% DM)	ADF (% DM)	DMI (%)	DDM (%)	RFV
NT	18.2	38.0	2.9	0.8	0.5	30.6	23.4	3.9	70.7	215.0
35S-SPS	24.2	41.0	3.7	1.2	0.7	23.1	18.8	5.2	74.3	299.5
% Change	32.6	43.1	27.6	48.1	25.0	−24.5	−19.8	32.5	5.1	39.3

5 mM NH₄NO₃	CP (% DM)	SoIP (% CP)	NPN (% DM)	NDIP (% DM)	ADIP (% DM)	NDF (% DM)	ADF (% DM)	DMI (%)	DDM (%)	RFV
NT	21.7	44.0	4.3	0.7	0.4	27.3	20.4	4.4	73.0	249.1
35S-SPS	25.4	37.0	5.7	0.8	0.5	18.8	14.7	6.4	77.4	383.0
% Change	17.2	−1.5	33.1	20.0	33.3	−31.0	−27.9	45.0	6.1	53.8

% Change NH₄NO₃:N₂	CP	SoIP	NPN	NDIP	ADIP	NDF	ADF	DMI	DDM	RFV
NT	18.7	37.4	46.9	−17.7	−25.0	−10.8	−12.9	12.1	3.3	15.8
35S-SPS	4.9	−5.4	53.2	−33.3	−20.0	−18.5	−21.7	22.6	4.3	27.9

Moreover, the percent of SoIP and the percent non-protein nitrogen (NPN) in these plants increased approximately 43 and 28 percent, respectively. The amount of protein (nitrogen) bound to fiber, defined as neutral detergent insoluble protein (NDIP) and acid detergent insoluble protein (ADIP), was generally low in both sets of plants (Table A.7). Nevertheless, a 25 and 48 percent increase in the values of ADIP and NDIP, respectively, was seen in the 35S-mzSPS transformed plants (FIG. 17A). Values determined by NDIP analysis comprise a fraction of the protein (nitrogen) in fiber that is both slowly degradable by rumen microbes and digestible in the small intestine of animals, while the values of ADIP analysis, a component of NDIP, represents protein (nitrogen) in fiber that is highly resistant to microbial and mammalian enzymes and consequently, completely unavailable to both. An estimate of the degradable/digestible fraction of protein (nitro-

tein bound to fiber. Moreover, the lower fiber content in the transformed plants translated into higher RFV content enhancing potential digestible dry matter intake when compared to the NT plants. These results validate the nutritional superiority of the 35S-mzSPS transformed plants over the NT plants when kept under symbiotic N_2-fixing conditions. Moreover, the reduction in fiber content shows that up regulation of SPS in alfalfa results in the reduction of cell wall synthesis or production of structural carbohydrates.

The 35S-mzSPS transformed plants have some characteristics that are similar to those seen in hyper-nodulating soybean plants, such as an increase in nodule number, darker green leaf pigmentation, and a reduction in shoot biomass. Hence, it was hypothesized that the alfalfa 35S-mzSPS transformed plants were diverting carbon, typically used for growth and development, to the root nodules to meet the

increased demand of carbon in these metabolically active carbon sinks. To test this hypothesis, another set of 20 NT and 35S-mzSPS transformed alfalfa plants were clonally propagated under greenhouse conditions. These plants, in contrast to the previous experiments, were kept under non-symbiotic N_2 fixing conditions by feeding them about 5 mM NH_4NO_3 and penicillin [100 µg·mL-1] in 0.5× Hoagland's nutrient solution. These experiments were conducted in parallel with the sets of plants grown under symbiotic N_2 conditions as described in previous sections.

and Table A.7 above, the percentage of CP and NPN was approximately 17 and 33 percent higher, respectively, in the transformed plants when compared to the NT plants. No substantial difference in the levels of SoIP was seen between the two sets of plants. While both ADIP and NDIP levels increased approximately 33 percent and 20 percent, respectively, in the transformed plants, the percent of degradable/digestible protein (nitrogen) bound to fiber was unchanged between the two sets of plants. Moreover, fiber content was approximately 30 percent lower in the ADF and NDF values

TABLE A.5

Plant growth (length) measurements of alfalfa non-transformed and 35S-mzSPS transformed plants grown under different nitrogen conditions.

Samples	Stem Length			Internode Length			Shoot:Root Ratio		
N_2 Fixing	Average	S.E.	t-test	Average	S.E.	t-test	Average	S.E.	t-test
NT	6.75	±0.07	0.04	1.22	±0.01	0.02	1.39	±0.02	0.00
35S-mzSPS	577	±0.6		0.90	±0.02		0.98	±0.02	
Change	−14.62			−26.30			−29.90		
5 mM NH_4NO_2	Average	S.E.	t-test	Average	S.E.	t-test	Average	S.E.	t-test
NT	7.84	±0.02	0.00	1.66	±0.00	0.00	1.13	±0.02	0.52
SPS	6.36	±0.02		1.07	±0.00		1.19	±0.01	
% Change	−18.89			−35.56			5.38		
% Change NH_4NO_2:N_2 Fixing	Stem Length			Internode Length			Shoot:Root Ratio		
NT	16.03			37.05			−16.88		
35-mzSPS	10.23			18.30			22.24		

Interestingly, quantitative analysis of stem and internode length showed similar trends with the exception of the shoot to root ratios (length) that showed no significant differences between the 35S-mzSPS transformed and NT plants (FIG. **18** and Table A.5; P=0.52). The total shoot and root lengths, however, were only measured once in the set of plants fed NH_4NO_3 and the measurement were done on the initial growth of the shoots after cuttings were established. Moreover, when comparing data of plants fed NH_4NO_3 with plants kept under symbiotic N_2 fixing conditions, only the 35S-mzSPS transformed plants demonstrated an increase of approximately 22 percent, in shoot to root ratios (Table A.5). In contrast to the transformed plants, the shoot to root ratios of the NT plants dropped approximately 19 percent in the set of plants fertilized with NH_4NO_3. Nonetheless, significant differences were seen in the average stem and internode length, of which, the 35S-mzSPS transformed plants were approximately 19 and 37 percent lower, respectively, than the NT plants. Shoots were cut back two consecutive times and similar trends were consistently seen in transformed plants. When comparing data of plants grown under the two different nitrogen regimens, the NT plants demonstrated a larger increase in stem and internode lengths of approximately 16 and 38 percent, respectively, when fed about 5 mM NH_4NO_3, while the 35S-mzSPS transformed plants showed about 10 and 18 percent increase. Moreover, the transformed plants fed NH_4NO_3 exhibited similar delays in flower emergence and changes in leaf pigmentation as previously described for the plants grown under symbiotic N_2 fixing conditions (Data not shown).

Because the set of NH_4NO_3 fed plants shared similar characteristics with plants kept under symbiotic N_2 fixing conditions, this set of plants was also analyzed for any changes in forage quality as previously described. As shown in FIG. **19**

which ultimately translated into a substantial increase, approximately 54 percent, in the RFV content of the NH_4NO_3 fed 35S-mzSPS transformed plants. It would thus appear that the NH_4NO_3 fed 35S-mzSPS transformed plants behaved similarly as the transformants under N_2-fixing condition with regards to phenotype, forage quality, and stem and internode length. Furthermore, when comparing data between the two nitrogen conditions (Table A.7), both set of plants, non-transformed and 35S-mzSPS transformed plants, demonstrated improved forage quality when fed NH_4NO_3. While a more substantial increase in RFV content and decrease in degradable/digestible protein bound to fiber was seen in the 35S-mzSPS transformed plants, the NT plants showed a higher increase in the levels of CP and SoIP when fed NH_4NO_3. Both set of plants shared similar increases in NPN when comparing data from the two nitrogen conditions. It is important to note, the 35S-mzSPS transformed plants kept under symbiotic N_2 fixing conditions were higher in RFV content and shared similar to slightly higher values in protein content (CP, SoIP, NPN, and degradable/digestible protein bound to fiber) when compared to the non-transformed plants under fertilized conditions (Table A.7). Taken together, the data indicates that the 35S-mzSPS transformants, under symbiotic conditions, have equal to or better forage quality than alfalfa fertilized with synthetic nitrogen.

Several independent transformants with the 35S-mzSPS gene construct (#3, 6, 7, 12, 13, 14, 15, 17, 18) along with six non-transformed (1-6) plants were transferred to pots and placed under greenhouse conditions. These plants were immediately inoculated with *S. meliloti* and fed N free Hoagland's media to initiate the symbiotic relationship between the host and the bacteria. Following exposure to the bacteria, it takes about 3 weeks for N_2-fixation to begin and the plants go through nitrogen stress during this period. Interestingly,

the transformed plants exhibited a phenotype not previously seen with the other sets of transformants which were first established by growing them with nitrogen. The newly emerged leaves in the shoot apical meristems of all the transformed plants were completely white and showed no signs of pigmentation (FIGS. **20** and A.3). Growth of the shoot apical meristems was monitored for about 11 days to observe the development and expansion of newly emerged trifoliate in the transformed plants. Pictures taken every two days of the shoot apical meristems of NT (#4) and 35S-mzSPS transformant (#12) are shown in FIG. **20** and represents what was seen in each class of plants. All the plants exhibited normal emergence and expansion of the new trifoliates. However, the trifoliates were green from the start of emergence in the NT plants. The pigmentation in the newly emerged trifoliate in the 35S-mzSPS transformed plants, began to slowly appear near the apex of each leaflet only upon completion of trifoliate expansion. Even then, 100% of the leaflets were not green, an effect seen as far down as the third or fourth fully expanded trifoliate from the shoot apex (FIG. **20**).

The major difference between the two sets of plants is the higher level of sucrose and its derivatives in the transformants under conditions of N deficiency compared to the control plants and this phenotype could be an attribute of this imbalance in C and N metabolites. To monitor the status of the C metabolic pathways, the plant apices of control plants and the transformants were stained for starch (Fig. A.4).

While the control plants showed staining for starch in the young emerging trifoliates, the same leaves in the transformed plants were not stained indicating the absence of starch synthesis, indicating that the extreme chlorosis seen in the apices of the transformants is due to the huge imbalance between sucrose and N, which affects the induction of many of the photosynthetic genes including those involved in the synthesis of the pigments and starch.

To check if N deficiency was indeed the basis for chlorosis in the emerging leaflets, the transformants were supplemented with about 5 mM NH_4NO_3. The pigmentation in most of the leaves of the 35S-mzSPS transformed plants was restored within approximately 20 days (FIG. **21**). The NT plants, when treated with about 5 mM NH_4NO_3 showed a more intense green color, likely because the leaves were already green to start with.

The complete N profile and fiber analysis of the above ground tissues of the two classes of alfalfa plants grown under the two different N conditions demonstrated that forage quality was enhanced in the shoots of the 35S-mzSPS alfalfa transformants (FIGS. **17** and **19**; Table A.7). The level of fiber, measured as ADF and NDF content, was substantially lower in shoots of the transformants, which corresponded to about 5-6% increase in digestible dry matter and up to about 45% increase in dry matter intake, respectively. Moreover, levels of protein, particularly CP, were substantially higher in the transformants. It is important to note that the leaves of alfalfa are more digestible and higher in nutrient and CP content than the stems. While the leaves showed only a slight decline in digestibility as the plant matured, levels of CP declined continuously from the vegetative to the early seed stage. Several key characteristics exhibited by the alfalfa 35S-mzSPS transformants seemingly influenced the above forage tests such as delayed flowering time/development, increased leaf chlorophyll and N content, and decreased stem internode length (FIGS. **18**, **20**, **21**, and **22**). The increase in soluble sugars resulting from increased sucrose biosynthetic capabilities of the transformants also has a role in the improvement of forage quality. The developmental stage of the plant is an important factor to influence forage quality. The tissue of plants in this

example were harvested before the plants entered the bud/bloom stage. However, the NT plants were still ahead developmentally when compared to the 35-mzSPS transformants. Moreover, the 35S-mzSPS alfalfa transformants under symbiotic conditions exhibited higher N protein profile and a higher potential digestible dry matter intake (RFV) value when compared to the NT plants kept under non-symbiotic, fertilized, conditions (Table A.7). These findings show that the 35S-mzSPS transformants perform better than the NT plants, particularly under greenhouse conditions.

Increasing SPS activity leads to increased sucrose synthesis and increases the leaf to stem ratio, photosynthetic rates, nodulation and nitrogen fixation, elemental N, chlorophyll and protein content. The flowering time is delayed implying a prolonged vegetative state. However, a positive attribute of increasing SPS activity is the improvement in forage quality. A major focus for forage breeding and biotech programs includes improving forage digestibility. A 10% increase in cell-wall/fiber digestibility of forages has been estimated to increase production of milk and beef production by 350 million dollars/yr, as well as decrease manure production by 2.8 MM tons/yr and decrease grain supplements by 2 million tons/yr. Forage legumes have provided a critical component of feed for livestock in the meat and dairy industries for centuries. In fact, the dairy industry in New Mexico relies heavily on alfalfa, the number one cash crop in the state, due to a combination of several factors, such as its high nutritional quality and yield production. Animal performance in the above industries depends in part on the nutritional value or forage quality of the dry matter the livestock consumes.

Example 2

Referring to FIGS. **29-31**, transgenic alfalfa plants transformed with the glutamine synthetase Gmglnβ1 showed improved growth, elongated stem, increased protein and enhanced nitrogen use efficiency. In FIG. **31**, alfalfa shoot height was measured, shoots were cut and fresh weight determined after five weeks of growth. Fresh tissues were dried out at about 37° C. and dry weight were measured. Asterisks indicate that the differences are statistically significant.

Example 3

An embodiment of the present invention was used to determine:

   i. if the 3'UTR mediated posttranscriptional regulation of GS1 is a universal phenomenon.

   ii. if the 5' and/or 3'UTR of the Gmglnβ1 gene play a role in translational regulation.

   iii. if the posttranscriptional regulatory step is mediated by a protein factor or miRNA.

   iv. the biochemical signal(s) that stabilizes/destabilizes the transcript and/or translation repression.

To check if translation enhancement by the Gmglnβ1 5'UTR was due to its interaction with some factor that is only present in the plant cells, in vitro reticulocyte and wheat germ translation systems were used to check for translational regulation of the Gmglnβ1 transcript with or without its 3' and 5'UTRs.

Referring to FIG. **32**A, pGSβ103, is a gene construct without the 5'UTR but with the 3'UTR, pGSβ153, has both 5' and 3'UTRs, pGSβ150 has the 5'UTR but not the 3'UTR, and pGSβ100 lacks both the 5' and 3'UTRs. The glnβ$_1$ 5'UTR was replaced by the mRNA leader of the CAMBIA CaMV 35S promoter and the 3'UTR was replaced by the NOS terminator. The isolation of the Gmglnβ1 gene (NCBI accession number

AF301590) was previously described. Four different GSβ1 gene constructs with and without its 5' and 3'UTRs were made. The Gmglnβ1 gene constructs either included its own 5'UTR (GSβ150, GSβ153), its own 3'UTR (GSβ103, GSβ153), or lacked both its 5' and 3'UTR (GSβ100). In GSβ150 and GSβ100, the 3'UTR was replaced with the NOS 3'UTR and in pGSβ103 and GSβ100, the 5' UTR was replaced with the mRNA leader of the CaMV35S promoter:: uidA gene construct in the CAMBIA 2301 plasmid (CAMBIA, Canberra, Australia). To make the gene constructs with the Gmglnβ1 5'UTR, the CaMV35S promoter was amplified, using pCAMBIA2301 plasmid as a template, with reverse primers with an EcoRI site downstream of the CaMV35S transcription start site (5'-GAG AAT TCC CGT GTT CTC TCC AAA TGA AAT GAA CTT C-3') (SEQ ID. NO. 6). The CaMV35S promoter for the Gmglnβ1 gene constructs without the 5'UTR was amplified with a reverse primer that included extra nucleotides encoding the first seven amino acids of the Gmglnβ1 gene, down to a BglII site (5'-AGA TCT GAG AGC GA GAC ATG GTC AAG AGT CCC CCG TGT T-3') (SEQ ID. NO. 7), which was used to clone the remaining of the Gmglnβ1 gene (FIG. 32b). The forward primer for amplification of the CaMV35S promoter included a HindIII at the 5' end (5'-AAG CTT CAT GGA GTC AAA GAT TC-3') (SEQ ID. NO. 8). The Gmglnβ1 gene constructs without its 3'UTR (GSβ150 and GSβ100) were made by removing the Gmglnβ1 3'UTR and replacing it with a SmaI/PstI NOS 3'UTR fragment amplified from the pMON316 vector (forward primer: 5'-CCC GGG ATC GTT CAA ACA TTT GGC AAT AAA GTT-3' (SEQ ID. NO. 9); reverse primer: 5'-GAG CTC TGC AGC CCG ATC TAG TAA CAT AGA CAC C-3') (SEQ ID. NO. 10). For the 3-glucuronidase gene construct GUSA50, the CaMV35S promoter comprising the Gmglnβ1 5'UTR was amplified from the GSβ153 gene construct by PCR using a primer comprising a NcoI site at the translation start site (FIG. 35, reverse primer: 5'-CCA TGG CTC CAA AAT CAA GCA AAG ACC CTT CTT T-3') (SEQ ID. NO. 11), which was then ligated to the uidA gene excised from the CAMBIA 2301 plasmid as a NcoI/BstEII fragment. A BstEII/PstI NOS 3'UTR fragment was amplified by PCR from the CAMBIA 2301 (forward primer: 5'-GGT GAC CAG CTC GAA TTT CCC CGA TCG TTC AAA CAT TTG GC-3') SEQ ID. NO. 12) and a NOS SacI/PstI reverse primer (as above) and placed at the end of the uidA gene. All PCR products were cloned into the pGEM-T easy vector and sequenced. Gene elements were assembled in the pBluescript II KS (–) plasmid. The constructs were sequenced and subsequently cloned in the pCAMBIA 2300 vector as KpnI/PstI inserts and transformed into *A. tumefaciens* strain GV3101 for plant infiltration.

Referring to FIG. **23**, the 3'UTR of MsGS100, like that of the Gmglnβ1 gene plays a role in transcript turnover. The 3'UTR of MsGS13, however, has a stabilizing effect on the transcript compared to the NOS 3'UTR. The two alfalfa GS1 genes exhibited differential posttranscriptional regulation mediated by their 3'UTRs. Thus, the 3'UTR mediated transcript turnover was concluded to not be a universal phenomenon for all GS1 genes.

Referring to FIG. **24**, the tissues infiltrated with the GSβ100 and GSβ150 constructs showed higher accumulation of GS1 polypeptide and a novel GS holoprotein. The tissues with the GSβ150 construct showed higher accumulation of the GS1 polypeptide and the novel holoprotein compared to tissues infiltrated with the GSβ100 construct. Tissues with GSβ153 showed a higher level of GS1 polypeptide compared to tissues infiltrated with the GSβ103 construct. The 3'UTR of Gmglnβ1 functions independent of the 5'UTR to

destabilize the transcript. While the 3'UTR of Gmglnβ1 gene plays a role in transcript turnover, the 5'UTR functions as a translation enhancer.

Referring to FIG. **25A**, differences in the intensity level with the NPTII probe in the different transformants reflects position effect due to random insertion of the T-DNA in the genome of the transformant. Note: transformants T13, T22, T37 (*) showed higher NPTII transcript level and relatively lower level of Gmglnβ1 transcript and transformants T19, T38, T49 (*) showed higher level of Gmglnβ1 transcript relative to the NPTII transcript. Transformants T13, T22, T37 besides exhibiting extreme silencing of the transgene, also showed additional hybridizing bands with the Gmglnβ1 and Gmglnβ1 3'UTR as probes, and can be used for understanding the mechanism of transcript turnover.

Referring to FIG. **25B**, a direct correlation between the hybridization signal with the NPTII probe and the Gmglnβ1 probe was observed in the GSβ150 transformants suggesting transcriptional regulation of the transgene. No direct correlation between the hybridization signal with the NPTII probe and the Gmglnβ1 probe was observed in the GSβ153 transformants. Gmglnβ1 transcript level is regulated by its 3'UTR at the level of transcript stability. RNA processing involves the formation of intermediates.

Referring to FIG. **26**, analysis of multiple clones from each set of alfalfa transformants showed that while RNA from T13 transformant used the same polyadenylation site at position 226 past the stop codon, the RNA from T19 transformant used multiple polyadenylation sites, at positions 194, 216 and 226 past the stop codon. 3' processing of the transcript may have a role in transcript instability. Only regions a and b were maintained in the mature transcript following polyadenylation and thus may be target regions for transcript turnover.

Referring to FIG. **27**, there is a direct correlation between the glnβ1 mRNA and GSM holoprotein irrespective of whether the transformant is GSβ153 or GSβ150. A direct correlation between transcript level and the corresponding protein irrespective of whether the transcript has or does not have its 3'UTR indicate that the Gmglnβ1 3'UTR does not cause repression of translation.

Referring to FIG. **28**, compared to control plants, the transformants showed major immunostaining in the vasculature of the leaves, stem and roots. Alfalfa transgenic plants showed higher amino acid concentration and higher accumulation of asparagine and proline. The higher accumulation of the amino acids in the stem over leaves of the transformants is an attribute of the vasculature enhanced expression of the CaMV 35S promoter. Increased GS activity is associated with changes in amino acid pools and other metabolites.

Agrobacterium tumefaciens strain GV3101 (Rif r) was used for tobacco infiltration experiments. Tobacco plants (*Nicotiana tabaccum* var. *Xanthi*) were propagated from cuttings of tops of plants grown under sterile conditions in magenta boxes containing solid Murashige and Skoog media supplemented with about 1% Sucrose. New tops were cut and transferred to new media every three weeks. Plant tops with developed roots were moved to pots with sterile vermiculite and grown for 3 weeks at a 16 h light/8 h dark cycle under fluorescent light before infiltration with *Agrobacterium*. Plants were maintained with Hoagland's nutrient solution containing about 5 mM NH₄NO₃.

Infiltration of *N. tabaccum Xanthi* leaves was done as a modification of a previous method. Single colonies of *A. tumefaciens* comprising the different gene constructs were grown overnight in LB media with Kanamycin and Rifampicin selection (about 50 µg/ml each). Cultures were diluted 10 fold in LB media and grown with antibiotic selection to a 0.5

OD600. Cells were centrifuged and resuspended in the same volume of induction media (about 10 mM MES, about 10 mM MgCl$_2$, about 0.2 mM acetosyringone, about pH 5.7) and incubated overnight at 30° C. with shaking. *Agrobacterium* cells were then centrifuged and resuspended in the same volume of infiltration media (about 1 mM MES, about 0.5 mM MgCl$_2$, about 0.02 mM acetosyringone, about pH 5.7). The three youngest leaves of each tobacco plant were uniformly infiltrated with the *Agrobacterium* suspension (two plants per gene construct) using about 1 ml disposable syringe without a needle. Four days after infiltration, the middle vein of the leaf was removed and the leaf blades were used for protein extraction or harvested in liquid nitrogen and frozen at about –80° C. for RNA isolation.

RNA was isolated from leaf tissues by phenol/chloroform extraction and LiCl precipitation as previously described. Total leaf RNA was subjected to 1.5% (w/v) formaldehyde-agarose gel electrophoresis followed by northern blotting using 32P-labeled DNA probes. The same blot was hybridized to the different probes following stripping before hybridizing with the next probe.

RNA secondary structure of the Gmglnβ1 5'UTR was predicted with the RNA folding by free energy minimization software MFOLD 2.0 and with the Unified Nucleic Acid Folding software UNAFold 3.8 with similar results.

Fresh leaf tissue was ground in a mortar with about 2 volumes of extraction buffer (e.g., 25 mM Tris, 20% glycerol, 5% ethylene glycol, 1 mM MgCl$_2$, 1 mM EDTA, 0.1% Triton X100, 5 mM DTT, 3% insoluble PVPP, pH 8). *E. coli* proteins were extracted in the same buffer without PVPP or Triton X100 and sonicated for five cycles of about 10 sec each. Plant and bacterial extracts were clarified by centrifugation at about 20,000×g for about 15 min at about 4° C. and transferred to new tubes. Protein concentration was determined colorimetrically with a protein assay solution (Bio-rad, Hercules, Calif.). Protein extracts were fractionated by non-denaturing or SDS polyacrylamide gel electrophoresis as described, followed by blotting to PVDF transfer membranes (Millipore, Billerica, Mass.). Proteins were immunodetected with antibodies raised against soybean GS1 or with commercially available anti-β-glucuronidase (GUS) antibody (ABCAM, Cambridge, Mass.) and anti-glutathione transferase (GST) antibody (GE Healthcare, Piscataway, N.J.) and a secondary antibody coupled to Alkaline Phosphatase. GS immunoreacting bands were quantified using Carestream Molecular Imaging Software 5.0 (Carestream Health, Rochester, N.Y.). A statistical analysis of the GS band intensities was performed (unpaired t test) using the Prism 5.0 software (GraphPad Software).

β-glucuronidase activity in extracts of infiltrated tobacco plants was determined using the fluorogenic substrate 4-methyl umbelliferyl glucuronide (4-MUG, Gold Biotechnology, Saint Louis, Mo.). Leaf extracts were incubated in a 1 mL reaction containing about 1 mM 4-MUG at 37° C. Aliquots (about 200 μL) were removed at about 10 min or about 30 min intervals, the reaction was stopped by adding about 1.8 mL of 0.2 M Na2CO3 and the fluorescence of 4-methyl umbelliferone (4-MU) produced was measured in a fluorometer (Turner Designs, Sunnyvale, Calif.) with an excitation wavelength at 365 nm, emission filter at 455 nm. GUS specific activity was calculated against a 4-MU standard curve (from about 0.1 to 5 μM).

Total RNA isolated from tobacco leaves infiltrated with the different GS1 gene constructs was reverse transcribed (RT) with an oligo-dT containing an anchor primer (5'-CCA GTG AGC AGA GTG ACG AGG AAG CTT(17)-3') (SEQ ID. NO. 13). GS1 PCR products were further amplified from the

cDNA with a forward primer for the GS1 5'UTR (5'-CAC GGG AAT TCT CTA AAA GAG ATC TT-3' (SEQ ID. NO. 14) for constructs GSβ150 and GSβ153), or with a forward primer for the CaMV35S mRNA leader (5'-AAC ACG GGG GAC TCT TGA CCA T-3' (SEQ ID. NO. 15) for constructs GSβ100 and GSβ103), and a reverse primer corresponding to the anchor sequence, which contained a Hind III site at the end to preserve a continuous polyA tail. GS1 PCR products were cloned in pCRBlunt II and sequenced. RNA was in vitro transcribed from the RT-GSβ1 clones with a T7 RNA synthesis kit and purified. Equal amounts of capped and polyadenylated RNA were translated in vitro in a wheat germ system and the products were analyzed by western blotting using GS1 antibodies as described in a previous section.

The plasmid pKSglnβ153 contains a full length soybean cytosolic glutamine synthetase cDNA (Gmglnβ1) as an EcoRI fragment cloned in the sense orientation with respect to the P lac promoter (FIG. **38***a*) of the pBluescript II KS (−) phagemid, whereas the plasmid pKSglnβ135 has the Gmglnβ1 cDNA insert in the antisense orientation. The plasmid pGEX::glnβ1 has the full length Gmglnβ1 cDNA in the glutathione transferase expression plasmid pGEX-4T-2 and was created by inserting the Gmglnβ1 cDNA as a transcriptional fusion in the Eco RI fragment at the end of the GST gene. The 5'UTR of the Gmglnβ1 gene introduces a stop codon producing a GST protein in which the last nine amino acids were replaced by a leucine (see, FIG. **38**B). The GS plasmids were transformed into *E. coli* XL1 Blue cells. Ten mL bacterial cultures were grown to an exponential phase. Expression of the Gmglnβ1 gene from the P lac in the pBluescript II KS or the P tac promoter in the pGEX-4T-2 plasmids was induced by incubating with 5 mM IPTG for about two hours. Cells were harvested by centrifugation at about 5,000×g for 5 minutes at about 4° C. Cell pellets were resuspended in about 1 mL of extraction buffer, protein was extracted and analyzed by Western blotting as described in the previous sections.

To check if the 5'UTR of a cytosolic GS gene has any role in regulating transcript stability or translation, gene constructs driven by the CaMV 35S promoter comprising a Gmglnβ1 coding region having or lacking its own 3'UTR and 5'UTR (FIG. **32**) were examined by transient expression assays by means of *A. tumefaciens* infiltration of tobacco leaves (*Nicotiana tabaccum* var. *xanthi*). Four days after infiltration, the accumulation of the Gmglnβ1 mRNA was analyzed (FIG. **33**). RNA isolated from the infiltrated leaves was subjected serially to northern blot analysis using the coding region of the Gmglnβ1 gene, the 3'UTR of Gmglnβ1 gene, the NOS terminator and the NPTII gene as probes. Control tobacco plants infiltrated with *A. tumefaciens* without any plasmid did not show hybridization to any of the DNA probes. The accumulation of the NPTII mRNA was monitored to determine the efficacy of the infiltration procedure and to standardize for the Gmglnβ1 mRNA accumulation levels. As seen in FIG. **33**, the level of hybridization in the leaf samples infiltrated with the different gene constructs showed some differences in hybridization with the NPTII probe, likely an attribute of the uniformity of the agroinfiltration, i.e. the number of cells that get the T-DNA during the process rather than position effect. The Gmglnβ1 coding region showed hybridization to all the samples infiltrated with the GS1 gene constructs, although to different levels (see, FIG. **33**). Referring to FIG. **33**A, for each example, two independent plants for each construct were sampled, total RNA was isolated from 3 individual leaves from each independent plant 4 days after agroinfiltration with *A. tumefaciens* containing gene constructs of the Gmglnβ$_1$ gene with and without its 5' and

3'UTRs (as described in FIG. **32**). About twenty μg of each sample was then fractionated by electrophoresis and subjected to northern blot hybridization. The blots were sequentially hybridized with ^{32}P labeled probes corresponding to the coding region and the 3'UTR of the Gmglnβ$_1$ gene, the *Agrobacterium* nopaline synthetase terminator (NOS 3'UTR) and the kanamycin resistance (NPTII) gene. The 28S rRNA stained by SYBR Gold is also shown as a control for loading Quantification and standardization of the mRNA band intensities (see, FIG. **33**B) showed that the Gmglnβ1 mRNA accumulation in leaves infiltrated with the gene constructs containing the 3'UTR (GSβ103 and GSβ153) was ten times lower compared to the mRNA accumulation levels of the Gmglnβ1 gene with the NOS 3'UTR (GSβ150, GSβ100). This indicates that the 3'UTR of the Gmglnβ1 gene downregulates mRNA levels at a post-transcriptional step, as has been demonstrated in stably transformed alfalfa and tobacco plants. As is also seen in FIG. **33**, there is no difference in the transcript level for Gmglnβ1 gene in tissues infiltrated with the Gmglnβ1 gene constructs containing its own 5'UTR compared to the corresponding gene fusions in which the GS1 5'UTR was removed and replaced with the 5' noncoding region of the uidA gene of the CAMBIA 2301 (GSβ150 compared to GSβ100 and GSβ153 compared to GSβ103, FIG. **32**). The 3'UTR of the Gmglnβ1 gene and the NOS terminator were used as probes to further distinguish between the transcript levels for the Gmglnβ1 gene in the different constructs. Hybridization with the 3'UTR and the NOS terminator as probes showed no significant difference in the intensity of the hybridization signal between the constructs with and without the 5'UTR, GSβ103 against GSβ153 and GSβ100 against GSβ150, respectively. These results indicate that the Gmglnβ1 5'UTR does not have a role in controlling GS1 transcript levels and that the 3'UTR functions independent of the 5'UTR, in controlling transcript turnover.

To check if the 5'UTR of the GS1 gene affects translation, a pattern was analyzed of GS protein accumulation and GS holoenzymes in tobacco leaves infiltrated with the different CaMV35S::Gmglnβ1 gene constructs in protein blots with antibodies against the soybean GSβ1 protein. GS is an octameric enzyme and can be either homomeric or heteromeric; based on its composition GS shows slight differences in the migration pattern on native gels. Control tobacco leaf GS1, in its native conformation migrated as a complex mixture of isoforms, probably representing different heteromeric forms (see, FIG. **34**A). Gmglnβ1 infiltrated samples showed another immunoreactive band in addition to the endogenous complexes seen in control samples, the level being relatively higher in the GSβ100 and GSβ150 infiltrated tissues compared to GSβ153 infiltrated tissues, in keeping with the corresponding transcript level (see, FIG. **33**). However, GSβ103 infiltrated samples showed a GS1 holoenzyme profile similar to that seen in control samples, because of the low level of expression of this gene construct. To look for quantitative differences in the steady state level of the Gmglnβ1 protein with the different gene constructs, the infiltrated tissue extracts were subjected to SDS PAGE followed by western blot analysis. As seen in FIG. **34**, the Gmglnβ1 protein comigrated with the endogenous tobacco GS1 in their denatured form. The GS1 immunoreactive band was quantified and the intensity was standardized against the average intensity of the GSβ153 gene construct, which comprises the unmodified Gmglnβ1 gene that includes its own 5'- and 3'-UTRs (FIG. **34***b*). The accumulation levels of the GS1 protein in the tissues infiltrated with gene constructs that lack the Gmglnβ1 3'UTR (GSβ100, GSβ150) was higher than the accumulation of GS1 in tissues infiltrated with the Gmglnβ1 constructs

comprising the Gmglnβ1 3'UTR (GSβ103, GSβ153), in keeping with the corresponding transgene transcript level (FIG. **33**). However, in spite of the fact that tissues infiltrated with GSβ150 and GSβ100 showed the same level of the transgene transcript, the GS1 accumulation levels showed differences that were statistically significant. The GSβ150 infiltrated tissues showed a 27% higher level of GS1 accumulation than GSβ100 infiltrated tissues. Similarly, the GSβ153 infiltrated tissues showed a significant 31% higher level of GS1 protein accumulation compared to GSβ103 infiltrated tissues, even though there was no difference in the level of transgene transcript accumulation. The GS2 protein levels in the denatured samples showed a reduction in the transformants compared to control plants. The same results were obtained in multiple experiments. The difference in the steady state level of the GS1 protein between tissues infiltrated with Gmglnβ1 gene constructs with or without its 5'UTR cannot be attributed to differences in transcript level (see, FIG. **33**) or to differential protein turnover since the coding sequence and hence the amino acid sequence is identical between the gene constructs. These results show that the 5'UTR plays a role in enhancing translation and that it functions independent of the 3'UTR.

To further demonstrate that the Gmglnβ1 5'-UTR has a role in enhancing translation, a gene construct was made with the 5'UTR of the Gmglnβ1 gene inserted between the CaMV 35S promoter and the β-glucuronidase reporter gene (uidA). The resulting gene construct GUSA50 and the parent CAMBIA 2301 plasmid, which comprises the uidA reporter gene driven by the CaMV 35S promoter (see, FIG. **35**A), were agroinfiltrated into tobacco leaves. Protein was extracted four days after infiltration and analyzed by western blotting with anti-β-glucuronidase (GUS) antibodies and measurement of its enzymatic activity (GUS activity). An immunoreactive band of 71 kDa, corresponding to the predicted size of the uidA gene product, accumulates to at least 10-fold higher accumulation level in the tissues infiltrated with GUSA50 than in the CAMBIA 2301 samples (see, FIG. **35**B), while the control samples infiltrated with the empty binary plasmid, CAMBIA 2300, did not show the corresponding band. GUS activity measurements showed that the inclusion of the Gmglnβ1 5'UTR in the 35SCaMV::uidA reporter gene construct increased the expression of GUS by 19.8-fold compared to the GUS expression in plants infiltrated with the original CAMBIA 2301 plasmid (FIG. **35***c*). The results suggest that the 5'UTR of the Gmglnβ1 gene functions as a translation enhancer independent of the coding region and the 3'UTR of the Gmglnβ1 gene.

Total RNA from tobacco leaves agroinfiltrated with the different gene constructs was reverse transcribed with an oligo-dT comprising an anchor primer, and PCR amplified with a forward primer for the Gmglnβ1 5'UTR (for constructs GSβ150 and GSβ153) or with a forward primer for the CaMV35S mRNA leader (constructs GSβ100 and GSβ103) and a reverse primer corresponding to an anchor sequence which comprises a Hind III site to preserve a continuous polyA tail. Gmglnβ1 PCR products were cloned and sequenced. RNA was then transcribed in vitro from the RT-GSβ1 clones and analyzed by subjecting equal amounts of the reaction products to electrophoresis followed by nucleic acid staining. No difference was observed in the transcript level for the different gene constructs (data not shown) indicating that the 3'UTR mediated transcript turnover does not take place in the in vitro system. Equal amounts of capped and polyadenylated RNA were then translated in a rabbit reticulocyte system and in a wheat germ system and equal amounts of the translation reaction products were analyzed by western

blotting using an anti-GS1 antibody. No difference in the accumulation of the GS1 protein band translated from the different GSM transcripts was seen either in the rabbit reticulocyte system (data not shown) or in the wheat germ system (see, FIG. **36**), indicating that the Gmglnβ1 5'UTR did not enhance translation in the in vitro system, neither the 3'UTR has an effect in the in vitro translation system.

Because of the role that the 5'UTR of the Gmglnβ1 gene plays in translation, the 5' UTR was sequenced and analyzed for any distinguishing features. The minimum energy folding of the Gmglnβ1 5'UTR shows a hairpin structure with the AUG start codon in an open unfolded region (see, FIG. **37**). A few features that are usually found in the non-coding regions of bacterial mRNA, were also found like a track of purines at position −20 to −32 relative to the start codon that has complementarity to the 16S rRNA in its mRNA 'track' region (see, FIG. **38**B). There is a partial Shine Dalgarno (SD) consensus sequence (GGAG) in the Gmglnβ1 gene at position −1 relative to the start codon. Immediately upstream of the track of purines in the Gmglnβ1 5'UTR, there is a sequence similar to the *E. coli* rrnB BoxA, which though a transcriptional anti-terminator, binds the ribosomal S1 protein and acts as a translational enhancer in *E. coli*. It has been shown, that S1 is the major component of the *E. coli* ribosome directly involved in mRNA selection. Thus, the Gmglnβ1 gene transcript could be translated in the bacterial cells.

The regions, similar to bacterial translational elements, found in the 5'UTR of the Gmglnβ1 gene transcript may enable its translation in a bacterial system, the expression was analyzed in *Escherichia coli* of the plasmids containing Gmglnβ1 in the sense (pKSglnβ153) or the antisense (pKS-glnβ135) orientation with respect to the P lac promoter. The expression of the Gmglnβ1 gene was induced by IPTG in exponentially growing *E. coli* XL1-Blue cultures. Induced *E. coli* proteins were extracted, subjected to SDS PAGE followed by western blotting using anti-GSM antibodies. As shown in FIG. **38**, the plasmid containing the full length Gmglnβ1 cDNA in the sense orientation (pKSglnβ153) expressed a protein that co-migrated with the GS1 band from a tobacco leaf sample infiltrated with the GSβ150 gene construct. The Mr of the GS1 protein produced in the bacterial system rules out the possibility of it being a β-galactosidase/GSβ1 protein fusion. Furthermore, sequence analysis showed no indication of a translational fusion with the lacZ structural gene. The results indicate that the Gmglnβ1 gene with its own 5'UTR is functional when placed under the control of a bacterial promoter, producing an mRNA that is translatable by the bacterial ribosomes.

To further confirm the potential of the Gmglnβ1 5'UTR in recruiting bacterial ribosomes, gene constructs were made with the glutathione transferase gene. The Gmglnβ1 gene with its 5'UTR and its 3'UTR was placed as a non-translational fusion downstream of the GST gene in the expression plasmid pGEX 4T 2 (FIG. **38**B). The gene construct was transcribed from the IPTG inducible P tac promoter resulting in a polycistronic mRNA encoding a GST protein, eight amino acids shorter, followed by the glnβ1 5'UTR that would direct the translation of the GSβ1 protein. As seen in FIG. **38**, both the GST and the GSβ1 proteins accumulate in the bacteria expressing the GST/GSβ1 constructs (pGEX::glnβ1) confirming that the Gmglnβ1 5'UTR is functional in recruiting ribosomes in a manner similar to an internal ribosomal entry site (IRES), allowing the expression of the Gmglnβ1 gene. The expression of the GST protein in the pGEX::glnβ1 gene constructs is not affected, except for the fact that the

GST protein is eight amino acids shorter. This is the first demonstration of the 5'UTR of an eukaryotic gene functioning as an IRES in *E. coli.*

The data presented indicates that the 5'UTR of the Gmglnβ1 gene is the determinant of translation enhancement and confirms earlier findings that the 3'UTR of the Gmglnβ1 gene controls the turnover of the transcript. A transient assay using agroinfiltration has allowed a simultaneous check of the expression of different gene constructs involving the 5' and 3'UTRs of the Gmglnβ1 gene at the level of transcript and protein accumulation. By testing gene constructs in different combinations, with and without the 3'UTR/5'UTR, it has been demonstrated that the role of the 5'UTR of the Gmglnβ1 gene in translational control and the 3'UTR in transcript turnover with both the UTRs functioning independent of each other.

Northern blot analysis using different probes, showed a 20-fold higher level of accumulation of the Gmglnβ1 transcript in the tissues infiltrated with the gene with the NOS terminator (pGSβ100 and pGSβ150) compared to tissues infiltrated with the Gmglnβ1 gene constructs containing its own 3'UTR (pGSβ103 and pGSβ153, FIG. **33**). No differences in transcript levels were found between the samples with the gene constructs with the Gmglnβ1 5'UTR compared to the samples with the gene constructs without the 5'UTR when hybridized with either the GS1 coding region or the 3'-specific regions. The 5'UTR of the Gmglnβ1 most likely did not play a role in the 3'UTR mediated turnover of the GS1 transcript.

Comparison of the amounts of GS1 protein in the infiltrated tissues showed a significantly higher level of the GS1 polypeptide, 2.5-fold the amount of the endogenous GS1 levels, in the tissues infiltrated with the pGSβ150 gene construct (see, FIG. **34**). The pGSβ150 gene construct showed about 27% higher GS1 protein accumulation than with the Gmglnβ1 gene without its 5'UTR (pGSβ100). Similarly, the pGSβ153 infiltrated tissues showed a higher level of GS1 polypeptide compared to the pGSβ103 samples, with no difference in the corresponding transgene transcript level. Besides its own coding region and 3'UTR, the 5'UTR of the Gmglnβ1 transcript when placed in front of the uidA gene showed a huge increase in translation of the reporter gene, further emphasizing the role of Gmglnβ1 gene in enhancing translation. The results support the notion that the 5'UTR of the Gmglnβ1 gene functions as a translation enhancer. The 5'UTR of the Gmglnβ1 gene functions in an autonomous manner and functions as a translation enhancer independent of the coding region and the 3'UTR of the Gmglnβ1 gene. The 20-fold increase in expression level of the uidA gene with the 5'UTR of the Gmglnβ1 gene compared to the expression level of the uidA gene with its own mRNA leader (see, FIG. **35**), is striking when compared to the expression of the Gmglnβ1 gene with its 5'UTR which accounted for only about 27 to 31% increase in total GS1 protein accumulation, compared to the gene constructs without the 5'UTR.

The translation efficiency of mRNA is likely affected by the stability of the 5'UTR. The stem-loop region of the tobacco psbA-5'UTR and the Omega leader of the TMV RNA are important determinants of mRNA translation that act as translation enhancers because of their stable secondary and tertiary structure. The predicted secondary structure of the 5'UTR of the Gmglnβ1 is a stable hairpin with a free energy of −27.1 kcal·mole-1 that could play a role in translation enhancement (see, FIG. **37**).

The 5'UTR of the Gmglnβ1 gene does not function as a translation enhancer in an in vitro translation system (see, FIG. **36**). Indicating that some kind of factors, not present in the cell free systems but present in the leaf cells, interact with

the 5'UTR of Gmglnβ1 gene to enhance translation rates ruling out the possibility of the 5'UTR acting as translational enhancer just based on its secondary structure.

Sequence analysis of the 5'UTR of the Gmglnβ1 mRNA showed a track of oligopyrimidines at −48 to −33 nucleotides upstream of the translation start codon (FIG. **32***b*).

Referring to FIG. **38**, plant glutamine synthetase genes have been expressed in *E. coli*. The GS1 and GS2 enzymes encoded by these genes are functional when expressed in bacteria, and their products correctly assemble into active native forms. However, in all cases the GS genes have been introduced as translational fusions in recombinant expression vectors. In this study example it was shown that GS1 mRNA is efficiently translated in bacteria when introduced as non-translational gene fusions, indicating that the eukaryotic 5'UTR of the Gmglnβ1 mRNA comprises the information to allow its translation in vivo in a prokaryotic system. Bacterial translational initiation involves the binding of the 30S ribosomal subunit to the ribosome binding site on the mRNA by base pairing between a Shine-Dalgarno (SD) region and the mRNA binding site (MBS) at the 3' end of the 16S rRNA. There is a partial Shine Dalgarno (SD) consensus sequence (GGAG) in the Gmglnβ1 gene at position −1 relative to the start codon (FIG. **32**), though SD region is normally found between positions −15 to −2 relative to the start codon. There is also a track of purines at position −20 to −33 relative to the start codon that has complementarity to the 16S rRNA in its mRNA 'track' region and may have a role in translation initiation by enhancing the efficiency of recognition of the mRNA by the ribosome. Crosslinking studies have indicated that the ribosome might interact with the mRNA via alternative sites outside the SD region and the start codon. Sequences in the 16S rRNA other than the MBS, crosslink with nucleotides both upstream and downstream of the start codon, supporting the existence of alternative mechanisms of ribosome recruitment. Analyses of a large number of mRNA sequences have shown that base pairing is not the only significant factor in determining the efficiency of the expression of the mRNA. Several mRNA cis-elements have a substantial effect on the translation efficiency possibly due to its involvement in the ribosome-mRNA recognition. An alternative mechanism involves cis-acting mRNA sequences that serve as targets for the mRNA-binding ribosomal protein S1. This protein is essential for the translational machinery of Gram negative organisms and ensures the translation of mRNAs from a wide variety of sources. Immediately upstream of the track of purines in the Gmglnβ1 5'UTR, there is a sequence similar to the *E. coli* rrnB BoxA, which though a transcrip-

tional antiterminator, binds the ribosomal S1 protein and acts as a translational enhancer. This region in the Gmglnβ1 5' UTR likely represents an efficient ribosomal S1 protein binding site. The translatability of the plant GS1 mRNA in *E. coli* indicates the working of a universal mechanism for translation initiation. The secondary structure of the 5'UTR likely prohibits translation initiation from other sites while leaving open the initiator site and allowing for the recognition by the ribosomes via the several ligands and this mechanism is universal, as shown for the GSβ1 5'UTR secondary structure (see, FIG. **37**).

Phylogenetic studies point to two classes of GS genes GSI and GSII. Although there are some exceptions, GSI class is found in prokaryotes, whereas the GSII class represent the functional GS genes in eukaryotes. In contrast to the endosymbiotic hypothesis which suggests the transfer of a prokaryotic gene to the nuclei in plants during the establishment of the endosymbiosis, the phylogenetic studies show that the GS genes involved in organelle function diverged long after the endosymbiosis from a common ancestor GSII type gene. It is thus unlikely that the remnants of translational elements in the plant GS which may function in *E. coli* are from bacterial origin. The presence of these translation initiation elements in the GS1 rather indicate that the mechanisms of translation initiation are conserved between prokaryotes and eukaryotes, as it has been shown.

The ability of the Gmglnβ1 5'UTR to enhance translation of a protein coding region, is of great biotechnological significance in that plant cells can be just as efficient in the synthesis of a foreign protein as bacterial cells. Moreover, plant cells are more ideal for proteins that undergo post-translational modification. The translational enhancement property of the Gmglnβ1 5'UTR on the reporter gene is also very useful for checking the functionality of weak promoters.

The preceding examples can be repeated with similar success by substituting the generically or specifically described reactants and/or operating conditions of this invention for those used in the preceding examples.

Note that in the specification and claims, "about" or "approximately" means within twenty percent (20%) of the numerical amount cited.

Although the invention has been described in detail with particular reference to these preferred embodiments, other embodiments can achieve the same results. Variations and modifications of the present invention will be obvious to those skilled in the art and it is intended to cover in the appended claims all such modifications and equivalents. The entire disclosures of all references, applications, patents, and publications cited above are hereby incorporated by reference.

SEQUENCE LISTING

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Phe Asn Pro Ser His Tyr Phe Val Glu Glu Val Val Lys Gly Val Asp
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Glu Ser Asp Leu His Arg Thr Trp Ile Lys Val Val Ala Thr Arg Asn
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Ala Arg Glu Arg Ser Thr Arg Leu Glu Asn Met Cys Trp Arg Ile Trp
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Ile Ser Ala Arg Arg Lys Glu Gln Glu Gln Val Arg Arg Glu Ala Thr
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Gly Glu Leu Ala Pro Val Glu Thr Thr Lys Lys Lys Phe Gln Arg Asn
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Phe Ser Asp Leu Thr Val Trp Ser Asp Asp Asn Lys Glu Lys Lys Leu
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Glu Leu Ala Arg Ala Met Ser Met Met Pro Gly Val Tyr Arg Val Asp
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Leu Phe Thr Arg Gln Val Ser Ser Pro Asp Val Asp Trp Ser Tyr Gly
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Thr Tyr Lys Ile Met Arg Arg Ile Glu Gly Glu Glu Leu Ala Leu Asp
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Arg Asp Asp Ile Asp Asp Met Ser Ala Gly Asn Ala Ser Val Leu Thr
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Thr Val Leu Lys Leu Ile Asp Lys Tyr Asp Leu Tyr Gly Ser Val Ala
545                 550                 555                 560

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Gln Lys Val Lys Thr Val Asp Glu Met Arg Glu Arg Leu Arg Met Arg
        930   935   940

Gly Leu Arg Cys His Ile Met Tyr Cys Arg Asn Ser Thr Arg Leu Gln
945   950   955   960

Val Val Pro Leu Leu Ala Ser Arg Ser Gln Ala Leu Arg Tyr Leu Ser
                965   970   975

Val Arg Trp Gly Val Ser Val Gly Asn Met Tyr Leu Ile Thr Gly Glu
            980   985   990

His Gly Asp Thr Asp Leu Glu Glu Met Leu Ser Gly Leu His Lys Thr
            995   1000   1005

Val Ile Val Arg Gly Val Thr Glu Lys Gly Ser Glu Ala Leu Val
    1010   1015   1020

Arg Ser Pro Gly Ser Tyr Lys Arg Asp Asp Val Val Pro Ser Glu
    1025   1030   1035

Thr Pro Leu Ala Ala Tyr Thr Thr Gly Glu Leu Lys Ala Asp Glu
    1040   1045   1050

Ile Met Arg Ala Leu Lys Gln Val Ser Lys Thr Ser Ser Gly Met
    1055   1060   1065

<210> SEQ ID NO 5
<211> LENGTH: 356
<212> TYPE: PRT
<213> ORGANISM: Soybean

<400> SEQUENCE: 5

Met Ser Leu Leu Ser Asp Leu Ile Asn Leu Asn Leu Ser Asp Thr Thr

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1               5                    10                   15

Glu Lys Val Ile Ala Glu Tyr Ile Trp Ile Gly Gly Ser Gly Met Asp
            20                  25                  30

Leu Arg Ser Lys Ala Arg Thr Leu Pro Gly Pro Val Ser Asp Pro Ser
            35                  40                  45

Glu Leu Pro Lys Trp Asn Tyr Asp Gly Ser Ser Thr Gly Gln Ala Pro
            50                  55                  60

Gly Glu Asp Ser Glu Val Ile Leu Tyr Pro Gln Ala Ile Phe Arg Asp
65                  70                  75                  80

Pro Phe Arg Arg Gly Asn Asn Ile Leu Val Ile Cys Asp Ala Tyr Thr
                85                  90                  95

Pro Ala Gly Glu Pro Ile Pro Thr Asn Lys Arg His Ala Ala Ala Lys
            100                 105                 110

Val Phe Ser His Pro Asp Val Val Ala Glu Val Pro Trp Tyr Gly Ile
            115                 120                 125

Glu Gln Glu Tyr Thr Leu Leu Gln Lys Asp Ile Gln Trp Pro Leu Gly
            130                 135                 140

Trp Pro Val Gly Gly Phe Pro Gly Pro Gln Gly Pro Tyr Tyr Cys Gly
145                 150                 155                 160

Val Gly Ala Asp Lys Ala Phe Gly Arg Asp Ile Val Asp Ala His Tyr
                165                 170                 175

Lys Ala Cys Ile Tyr Ala Gly Ile Asn Ile Ser Gly Ile Asn Gly Glu
            180                 185                 190

Val Met Pro Gly Gln Trp Glu Phe Gln Val Gly Pro Ser Val Gly Ile
            195                 200                 205

Ser Ala Gly Asp Glu Ile Trp Ala Ala Arg Tyr Ile Leu Glu Arg Ile
            210                 215                 220

Thr Glu Ile Ala Gly Val Val Val Ser Phe Asp Pro Lys Pro Ile Lys
225                 230                 235                 240

Gly Asp Trp Asn Gly Ala Gly Ala His Thr Asn Tyr Ser Thr Lys Ser
                245                 250                 255

Met Arg Glu Asp Gly Gly Tyr Glu Val Ile Lys Ala Ala Ile Asp Lys
            260                 265                 270

Leu Gly Lys Lys His Lys Glu His Ile Ala Ala Tyr Gly Glu Gly Asn
            275                 280                 285

Glu Arg Arg Leu Thr Gly Arg His Glu Thr Ala Asp Ile Asn Thr Phe
            290                 295                 300

Leu Trp Gly Val Ala Asn Arg Gly Ala Ser Val Arg Val Gly Arg Asp
305                 310                 315                 320

Thr Glu Lys Ala Gly Lys Gly Tyr Phe Glu Asp Arg Arg Pro Ala Ser
                325                 330                 335

Asn Met Asp Pro Tyr Val Val Thr Ser Met Ile Ala Asp Thr Thr Ile
            340                 345                 350

Leu Trp Lys Pro
            355


<210> SEQ ID NO 6
<211> LENGTH: 37
<212> TYPE: DNA
<213> ORGANISM: Soybean and CaMV 35S

<400> SEQUENCE: 6

gagaattccc gtgttctctc caaatgaaat gaacttc                          37
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<210> SEQ ID NO 7
<211> LENGTH: 39
<212> TYPE: DNA
<213> ORGANISM: Soybean and CaMV 35S

<400> SEQUENCE: 7

agatctgaga gcgagacatg gtcaagagtc ccccgtgtt                           39


<210> SEQ ID NO 8
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Soybean and CaMV 35S

<400> SEQUENCE: 8

aagcttcatg gagtcaaaga ttc                                            23


<210> SEQ ID NO 9
<211> LENGTH: 33
<212> TYPE: DNA
<213> ORGANISM: Soybean and CaMV 35S

<400> SEQUENCE: 9

cccgggatcg ttcaaacatt tggcaataaa gtt                                 33


<210> SEQ ID NO 10
<211> LENGTH: 34
<212> TYPE: DNA
<213> ORGANISM: Soybean and CaMV 35S

<400> SEQUENCE: 10

gagctctgca gcccgatcta gtaacataga cacc                                34


<210> SEQ ID NO 11
<211> LENGTH: 34
<212> TYPE: DNA
<213> ORGANISM: Soybean and CaMV 35S

<400> SEQUENCE: 11

ccatggctcc aaaatcaagc aaagaccctt cttt                                34


<210> SEQ ID NO 12
<211> LENGTH: 41
<212> TYPE: DNA
<213> ORGANISM: Soybean and CaMV 35S

<400> SEQUENCE: 12

ggtgaccagc tcgaatttcc ccgatcgttc aaacatttgg c                        41


<210> SEQ ID NO 13
<211> LENGTH: 27
<212> TYPE: DNA
<213> ORGANISM: Tobacco leaves

<400> SEQUENCE: 13

ccagtgagca gagtgacgag gaagctt                                        27


<210> SEQ ID NO 14
<211> LENGTH: 26
<212> TYPE: DNA
<213> ORGANISM: Tobacco leaves

<400> SEQUENCE: 14

cacgggaatt ctctaaaaga gatctt                                         26
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<210> SEQ ID NO 15
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Tobacco leaves

<400> SEQUENCE: 15

aacacggggg actcttgacc at                                           22


<210> SEQ ID NO 16
<211> LENGTH: 281
<212> TYPE: RNA
<213> ORGANISM: Soybean and CaMV 35S

<400> SEQUENCE: 16

ugagcaaaac cugcauguuu ucucccuuuu gauggaaagg aacaguuaug cuuuucuuag    60

uaggauuugg ucucucucuc uuuuuaccuu uugauuggua cuaugguugg ugccuuguug   120

guuggugcaa cuaacuggca aggguuguuc auuguuuucu ucuauuccuu ucccucguuu   180

uccgauuguu acaaugacaa uaauuuaaug guuauuauca gucuuugaaca aagaaaugcu   240

gauugugaag uauaauaaua auauaugaaa uugccggaau c                      281


<210> SEQ ID NO 17
<211> LENGTH: 85
<212> TYPE: DNA
<213> ORGANISM: Soybean and CaMV 35S

<400> SEQUENCE: 17

gaattctcta aaagagatct ttttctgctc tttgaagaaa gaagggtctt tgcttgattt    60

tggagatgtc tctgctctca gatct                                        85


<210> SEQ ID NO 18
<211> LENGTH: 38
<212> TYPE: DNA
<213> ORGANISM: Soybean and CaMV 35S

<400> SEQUENCE: 18

cacgggggac tcttgaccat gtctctgctc tcagatct                           38


<210> SEQ ID NO 19
<211> LENGTH: 85
<212> TYPE: RNA
<213> ORGANISM: Soybean and CaMV 35S

<400> SEQUENCE: 19

gaauucucua aaagagaucu uuuucugcuc uuugaagaaa gaagggucuu ugcuugauuu    60

uggagauguc ucugcucuca gaucu                                        85


<210> SEQ ID NO 20
<211> LENGTH: 86
<212> TYPE: DNA
<213> ORGANISM: Soybean and CaMV 35S

<400> SEQUENCE: 20

ggatccccag gaattctcta aaagagatct ttttctgctc tttgaagaaa gaagggtctt    60

tgcttgattt tggagatgtc tctgct                                       86
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What is claimed is:

1. A method of producing an improved leguminous plant, plant cell or plant part with enhanced nitrogen use efficiency, and reduced flowering as compared to a corresponding non transformed leguminous wild type plant, plant cell or plant part comprising:

transforming a plurality of leguminous plants, plant cells or plant parts with an expressible nucleic acid sequence encoding the sucrose phosphate synthase (SPS) gene of maize, wherein the nucleic acid shares at least 95% sequence identity with SEQ ID NO: 1; and

selecting from the transformed leguminous plants, plant cells or plant parts a leguminous plant, plant cell or plant part having increased nitrogen use efficiency, and reduced flowering as compared to a corresponding non transformed leguminous wild type plant, plant cell or plant part.

2. The method of claim 1 wherein the nucleic acid has 100% sequence identity with SEQ ID NO: 1.

3. The method of claim 1 wherein the nucleic acid sequence is under the control of a promoter.

4. The method of claim 3 wherein the promoter is CaMV 35S.

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